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EFFECT OF X RAYS ON HEMOLYSIN FORMATION FOLLOWING VARIOUS IMMUNIZATION AND IRRADIATION PROCEDURES

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The effect of heavy total body X irradiation has been studied before and after a single intravenous injection of sheep red cells in rabbits (W. H. and L. G. Taliaferro and E. Janssen, 1952) as well as during the decline of hemolysin after a series of repeated injections (W. H. and L. G. Taliaferro, 1950b). In the work reported in 1952, we found two types of X-ray injury to hemolysin formation which could be correlated with the time of administering a single intravenous injection of approximately 1.25 ml of 1% red cells. The first one was found in animals receiving antigen 4 days before to 6 hours after 600 or 700 r X irradiation and consisted of a retardation in the appearance of hemolysin as well as a reduction in the rate of hemolysin accumulation in the blood, but normal or above normal peak titers were eventually attained. The above normal amounts of antibody were formed most frequently in animals irradiated within several hours immediately following the injection of red cells. The second effect was found in animals injected with antigen 12 hours to 7 days after irradiation and consisted of a marked and at times complete suppression of antibody which, in animals forming measureable amounts of hemolysin, was associated with an even more pronounced lengthening of the induction period and slowing of the rate of accumulation in the serum than in the first effect. In our previous paper (1952), we designated increased antibody formation when antigen was injected shortly before X rays as stimulation although it was associated with a slowing of the process. In this paper, we have termed it an enhancement and have found further that, with small amounts of X rays, it may occur with a normal or possibly an increased rate of antibody accumulation.

In the present work, we have studied both the enhancement and inhibition of antibody formation (1) when total body X irradiation was held relatively constant (500 or 600 r) and the amount of antigen was varied (table 1) and (2) when the immunization procedure was held constant (a single injection of 1.25 ml of 1% red cells/kg rabbit) and the total body X irradiation was varied (10r to 700 r in table 3). The single antigen injection was given intravenously 1 to 4 hours before irradiation to study enhancement and 2 days after irradiation to study inhibition by X rays. These times were selected because they were, for both enhancement and suppression,

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the approximate periods of greatest effect as found in our study with Janssen (1952). In addition, total body X irradiation was held relatively constant (500 or 600 r) while the immunization procedure consisted of 9 or 10 repeated intravenous antigen injections during an interval of 15 to 17 or $3\frac{1}{3}$ days (table 2). The first injection of the series was given (1) either just before irradiation or (2) 2 days after irradiation.

The work was undertaken primarily to form a necessary base line for further work on the mechanism of X-ray effects on antibody formation. The most interesting aspect of the work is the calculation of approximate dosage-response curves for both antigen and X-ray dosage. A secondary aim was to restudy the antibody enhancement already reported by us. Such corroboration is especially desirable because, in our own experience, the unknown variables in this type of work are so numerous that with the most careful attention to an exact repetition of experimental details, X-ray experiments at different times yield different results. The present results have been obtained over a period of 4 years, and most series represent a pool of rabbits treated at various times. Besides giving evidence on the effect of increasing the antigen dosage, the study of X irradiation in animals receiving multiple antigen injections is important as it more nearly corresponds to the situation in infections. The present work has been reported preliminarily (1953). It is partly cumulative since it includes some rabbits in experiment 2 from our earlier (1952) investigation.

Studies by other investigators on the effect of X rays on immunity were reviewed by us in 1951 and with Janssen in 1952.

MATERIALS AND METHODS

Materials and methods have been previously described (see especially W. H. and L. G. Taliaferro, 1950, and W. H. and L. G. Taliaferro and Janssen, 1952). Only a few salient details will be noted here.

The antigen for injecting and for the in vitro titrations consisted exclusively of sheep red cells. All suspensions of red cells for injection and for the in vitro titrations were standardized in terms of hemoglobin liberated by distilled water hemolysis such that 1 ml of the suspension plus 4 ml distilled water gave a reading of 150 on the Klett Summerson photoelectric colorimeter with a number 54 filter (approximate spectral range of 500 to 570 mμ). Such a suspension is called for convenience a 1% suspension or approximately 1.6×10^8 cells/ml. Also for convenience, all amounts injected are usually designated in terms of a 1% suspension although the amount never exceeded 30 ml. Washed cells were suspended in 0.85% sodium chloride solution and were always injected into the right ear vein per kg rabbit. The red cells from the several sheep used for titrations and injections were found to react similarly in in vitro hemolysisn tests.

All hemolysin titers were obtained in 50% units. A 50% unit is the amount of serum causing 50% hemolysis of 2 ml of 1% sheep cells, standardized as noted above, in the presence of four 50% units of complement. The titers are shown in figures 2 to 5 as amounts of serum and elsewhere reciprocally as the number of 50% units per ml of serum. They were read from plots according to the von Krogh equation of the amount of serum against the degree of hemolysis.

Our extensive studies on hemolysin formation (1950, 1951b and 1952) indicate that, following the induction period, the rise and fall of antibody can be approximated in most rabbits by a series of discontinuous curves described by the well known integral

$$A_t = A_0 e^{kt}$$

in which A_t and A_0 represent antibody titers at times t and 0, respectively, and k represents the rate of rise of the serum antibody. As a measure of the amount of antibody formed, we have used peak titer as obtained from fitted curves of log serum antibody against time (see fig. 2 and 3). This is probably the best single determination for antibody formation (see our discussion in 1952) because it is convenient to use and yields approximately the same conclusions as obtained from calculations for the total antibody formed. As measures of the rate of the immune process, we have used (1) the length of the induction period, i.e., the time elapsing between the injection of antigen and the first rise of antibody above the preimmunization level, and (2) the

rate of increase (k) of the initial rise (equivalent to the entire rise to peak titer when there was no change in rate), and (3) a derived or average k when the initial rise consisted of more than one phase. The derived k was obtained by subtending a line from the preimmunization titer at the beginning of the appearance of antibody to peak titer (see fig. 2 and 3).

Total body irradiation was used exclusively. The constants for the three machines were as follows:

Location of machine at the University of Chicago	Kv	Ma	Filter		Half layer value of filtered beam (mm copper)	Target distance from center of rabbit in cm	Direction of beam	r/min
			Cu (mm)	Al				
USAF Radiation	250	15	0.25	1	0.9	75	Horizontal	30–35
Dept. Anatomy	250	15	0.5	1	1.46	92	Vertical	15–16
Argonne Cancer	250	22	0.5	1	1.43	79	Vertical	32–35

All three machines were used to irradiate the rabbits in experiments 1 through 7 and 15 through 17. The Argonne Cancer Hospital machine alone was used for experiments 8 through 14. Two rabbits were irradiated at a time in aluminum boxes and were turned at the midpoint. The stage holding the rabbits revolved in front of the machine of the U. S. Air Force Radiation Laboratory. Variations in results were no greater between the 3 machines than with anyone at different times.

The rabbits were chiefly supplied by Mr. Harold Swift of Lakeside, Michigan, and were developed from 4 stocks originally supplied by Johns Hopkins University. A smaller number were obtained from the National Laboratory Animal Co. at Creve Coeur, Missouri. Both of these strains were remarkably free of snuffles or other respiratory diseases. A few additional animals were obtained from a local animal dealer. Males and females occurred in about equal numbers. They were kept in our laboratory for a week or more before being used and were fed on Dixie rabbit pellets from the Dixie Mills Co. at St. Louis, Missouri. At the start of an experiment they usually weighed between 2 and 2.5 kg. As far as we could determine, antibody formation was similar in rabbits from the various sources and, in any case, each experimental group was controlled with rabbits from the same source. The high mortality among the animals receiving 700 r and, to a less extent, 600 r may have been in part due to the fact that these doses were administered to rabbits which were obtained from the local dealer and which had a higher rate of respiratory infection and of *Eimeria perforans* (see Taliaferro et al, 1952).

For the statistical analysis of the results, log peak titers were used, while rates and times were treated arithmetically. This procedure is logical since changes in serum titer occur exponentially whereas rates and time are exponents. In addition, frequency distributions of arithmetic peak titers, although yielding a smooth curve, were markedly skewed, whereas frequency distributions of log peak hemolysin titers were more symmetrical (fig. 1), and the variability in individual series was much reduced. Thus, the coefficients of variation for the arithmetic peak titer of control series 3, 6, 9 and 12 (table 1), which received a single antigen injection, were 75, 87, 55 and 85%, respectively. After logarithmic transformation, the coefficients were reduced, respectively, to 16, 10, 7 and 11%. The same was true for the 3 control series 15, 17 and 20 (table 2) receiving repeated injections. The coefficients of variation for the arithmetic peak titers were 64, 52 and 51%, respectively, and, after logarithmic transformation, were reduced to 7, 10 and 7%, respectively. In the tables, besides the mean log values with standard errors, peak titers are shown as geometrical means (antilogs of mean log peak titers) and arithmetic mean peak titers ± standard

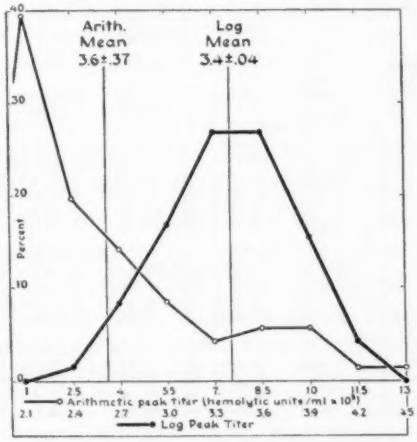

FIG. 1.—Frequency polygons for arithmetic and log peak hemolysin titers in control series 6 consisting of 71 rabbits.

errors. The last are included for comparison with data in our earlier papers.

Our method of handling peak titers is similar to that of Jensen who, according to Madsen and Rasch (1938), routinely used logarithmic and geometric means to characterize the antitoxin response of rabbits immunized with diphtheria toxoid. He assigned a value of 0.0005 antitoxin units/ml when the titer was too small to measure. Somewhat similarly, we have assigned the preimmunization titer to irradiated rabbits showing no rise. Animals having less than 10 50% units /ml were assigned this figure as it was the low limit of titratable antibody used in this work. Parenthetically, it should be noted that anergic rabbits only occurred in X-rayed groups.

Madsen and Rasch (1938) have criticized Jensen's method as not quite satisfactory and have plotted the accumulative probit of percentage of rabbits against the log antitoxin response. In several series, this plot yielded a section of a hyperbola. This hyperbola had two asymptotes which they believe are expressions of the two extremes, the one representing the fact that some animals do not produce any antitoxin and the other representing the upper limit to the amount of antitoxin any rabbit can produce under any conditions. We do not question the biological conclusions of these authors, but the probit method of determining median titers offered no particular advantage in our work because a probit plot against log peak titer gave straight lines in the range of 10 to 80% of the rabbits for any group, with very little deviation from linearity above and below these values. There was no indication of the hyperbolic sigmoid found by Madsen and Rasch. As would be expected from the slight skewness shown in figure 1 for the log peak antibody titers, the median titer calculated by the probit method was somewhat lower than the geometric mean. Thus, for series 6, consisting of 71 rabbits, the median response was 1800 units as contrasted to the geometric mean of 2500 units/ml.

EXPERIMENTAL RESULTS

Data on the antibody response in 475 rabbits used in the present study are divided into 17 experiments. Each experiment is further divided into 3 series: (A) irradiated rabbits receiving a single antigen injection or the first of a series of antigen injections 1 to 4 hours before X rays, (B) irradiated animals receiving antigen 2 days after X rays, and (C) unirradiated controls receiving antigen alone. Two exceptions occur. The *A* series in experiment 6 and the *B* series in experiment 8 were omitted because they were considered unnecessary.

The relation of antigen dosage (single injection) to X-ray modification of the antibody response.—Table 1 shows the effect of varying antigen dosage while

TABLE 1—*Primary hemolysin response† following one intravenous injection with sheep red cells (A) 1 to 4 hours before or B) 2 days after 500 r or 600 r total body irradiation along with (C) controls.*

Exp.	series	Ml 1% red cells per kg	Mean peak titer: Log	Mean peak titer: Geometric ×10³	Mean peak titer: Arithmetic ×10³	Rate of accumulation k: First phase	Rate of accumulation k: Derived when > one phase	Length in days of: Induction period	Length in days of: Antibody rise to peak	Number of rabbits
					A. One injection 1 to 4 hours before X rays					
1	1	0.125	3.75 ± .14+	5.50	8.08 ± 1.87*	0.9 ± .08	0.6 ± .05+	7.5 ± 0.7*	10.5 ± 0.8*	10
2	4	1.25	3.86 ± .08*	7.25	9.69 ± 2.12*	0.9 ± .07*	0.7 ± .04*	5.7 ± 0.4*	9.5 ± 0.5*	20
3	7	10	4.05 ± .10*	11.20	14.19 ± 3.11+	1.3 ± .10	0.9 ± .17	7.0 ± 0.5*	7.9 ± 1.4*	10
4	10	250	4.10 ± .08*	12.59	14.24 ± 1.66+	1.2 ± .09+	1.0 ± .09	5.4 ± 0.3*	7.4 ± 0.6	7
					B. One injection 2 days after X rays‡					
1	2	0.125	1.68 ± .08*	0.048	0.06 ± 0.01*	0.3 ± .03*	0.2 ± .02*	17.3 ± 2.5*	10.0 ± 1.9*	7 (10)
2	5	1.25	1.81 ± .11*	0.064	0.11 ± 0.02*	0.6 ± .20*	0.3 ± .04*	12.6 ± 1.0*	8.3 ± 1.7*	12 (6)
3	8	10	2.09 ± .14*	0.125	0.37 ± 0.08*	0.6 ± .11*	0.4 ± .07*	10.8 ± 1.7*	9.1 ± 2.0*	9 (3)
4	11	250	2.61 ± .18*	0.41	0.78 ± 0.31*	0.8 ± .19*	0.5 ± .11+	7.9 ± 0.2*	7.9 ± 1.2*	9
					C. Red cells alone: no X rays					
1	3	0.125	3.25 ± .09	1.78	2.82 ± 0.39	1.1 ± .08	1.0 ± .08	3.7 ± 0.19	5.2 ± 0.47	31
2	6	1.25	3.40 ± .04	2.52	3.60 ± 0.37	1.3 ± .05	1.2 ± .05	3.2 ± 0.11	4.3 ± 0.16	71
3	9	10	3.43 ± .06	2.70	3.21 ± 0.42	1.4 ± .10	1.1 ± .20	2.7 ± 0.14	5.0 ± 0.44	18
4	12	250	3.61 ± .08	4.08	5.54 ± 0.93	1.9 ± .08	1.2 ± .12	2.6 ± 0.19	5.4 ± 0.57	26

† All values are means ± standard errors. Data from individual rabbits were obtained from fitted curves. Numbers in parenthesis give additional rabbits which showed no antibody response. Mean peak titers were calculated from the total sample: other values only from those rabbits which showed an antibody response. The significance of the differences between each irradiated group and the corresponding control group are indicated by the following probabilities:
* $P \leq 0.001$ + $P = 0.002$ to 0.01 * $P = 0.02$ to 0.05

‡ Antigen given either one or two days after X rays in series 2 and 5.

holding irradiation at 500 or 600 r. This table also gives standard errors and statistical significances of the differences between all irradiated series and corresponding controls. The results with 500 and 600 r were pooled because there was no significant difference in the log mean peak titer of these two groups (cf. exp. 15 and 16 in table 3) and because both were in the range of maximum suppression (see later discussion).

When a single antigen injection was made 1 to 4 hours before irradiation, the geometric mean was invariably higher than in the unirradiated controls. Furthermore, the enhancement was progressively larger as the amount of antigen was increased in the first three doses (series 1, 4 and 7) and then remained constant (series 7 and 10). Thus, the differences between the geometric mean in each irradiated series over the corresponding control group were: 3720 units/ml in experiment 1, 4730 units/ml in experiment 2 and 8500 and 8510 units for experiment 3 and 4, respectively. All of these differences were significant: P for the smallest antigen dose was 0.02 and for the rest was <0.001.

Slowing of the rate of the immune process was indicated by a significantly longer induction period ($P<0.001$ in all doses). The derived value of k was only significantly lower for the two smaller antigen doses and the antibody rise was only significantly longer for the three smaller doses, but, in general, the values varied with the increase in antigen dose. Taken as a paired series of irradiated and control groups, Student's t test gave $P=0.05$ for the value of k and $P=0.01$ for the length of rise to peak titer.

These results indicate that, although enhancement occurred with all doses of antigen when injected 1 to 4 hours before 500 or 600 r total body irradiation, an antigen dose of 10 ml of 1% or larger gave the greatest enhancement of peak titer, whereas the smaller doses gave the most significant evidence of a retardation in the rate of the immune process. This is what would be expected from the effect of antigen dosage on peak titer and rate of the immune response (see Discussion).

When antigen was injected 2 days after 500 or 600 r total body irradiation, suppression of antibody formation occurred throughout the 2000-fold range of antigen dosage. Thus, there was a highly significant decrease in the amount of antibody formed as indicated by log peak titers ($P\leqq0.001$ for all doses). Concomitantly, there was a marked retardation of the immune process in those animals which formed measurable amounts of antibody, as indicated by a significant lengthening of the induction period and rise to peak titer and a decrease in the initial and derived values of k.

The amount of antigen injected apparently modified the degree of X-ray suppression of antibody formation. Thus, the mean log peak titer was significantly higher in the series receiving 250 ml of 1% than in those receiving 0.125 ml of 1% cells/kg ($P<0.001$). Differences between intermediate series were not significant, but there was a consistent increase in titer as the amount of antigen was increased, from a geometric mean of 48 units/ml in series 2 to 410 units/ml in series 11. As mentioned previously, those rabbits receiving antigen 2 days after irradiation, which formed no measureable antibody, were assigned their preimmunization titer in calculating mean peak titers. Their number in each group is indicated in parenthesis in the tables. The data in table 1 indicate that the percentage of such animals also increased as the size of the antigen dose decreased. Thus, in series 11 receiving 250 ml of 1% cells/kg, no completely negative rabbits oc-

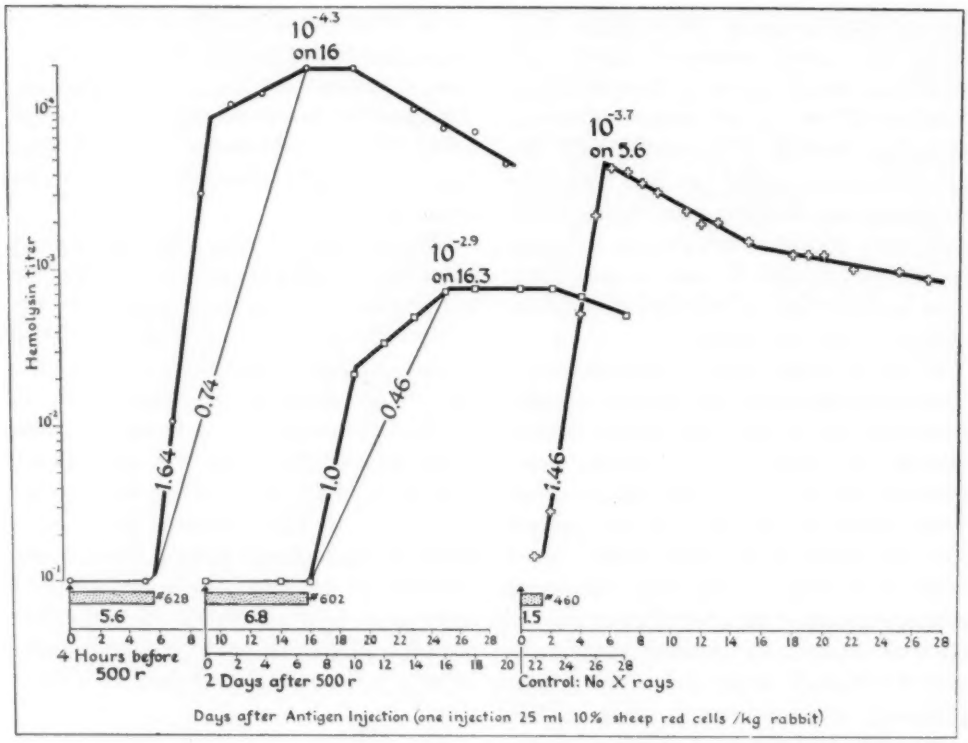

FIG. 2.—Representative examples of series 10, 11 and 12 in experiment 4 showing the hemolysin curve from 3 rabbits given 1 injection of 25 ml of 10% red cells/kg 4 hours before or 2 days after 500 r, and of an unirradiated control.

curred among 9 tested, whereas in series 2, receiving 0.002 of this dose, there were 10 out of a group of 17. The values of k were consistently lower and the length of the induction period longer as the antigen dose was decreased. This again indicates that suppression became more marked as the antigen dose was decreased.

The various changes in the X-irradiated rabbits as compared to the controls are exemplified in figure 2, which is a graphic representation of three rabbits from series 10, 11 and 12 of experiment 4. Here, as compared to control rabbit 460, which reached a peak titer of 5000 units after an induction period of 1.5 days and an initial rise during 4.1 days at a k rate of 1.46, the rabbit injected 4 hours before 500 r had a longer induction period but eventually a higher peak titer (20,000 units), whereas the rabbit injected 2 days after 500 r attained a lower peak titer (790 units) with all measures indicating a retarded rate for the immune process.

The relation of antigen dosage (repeated injections) to X-ray modification of the antibody response.—Table 2 shows the effect of serial antigen injections in a first group, of which one injection was given before and the rest after irradiation, and in a second group, of which all injections were given after the same amount of irradiation. The third group contain nonirradiated controls. This table also gives standard errors and statistical significances between each irriadiated and corresponding control series. Immunization by multiple anti-

TABLE 2.—*Hemolysin response† following 9 or 10 intravenous injections with sheep red cells during 15 to 17 days.‡ In section A, the first injection was given 1 to 4 hours before and, in section B, 2 days after 500 or 600 r total body irradiation. Controls in section C were not irradiated.*

Exp. and series		Ml 1% red cells per kg	Mean peak liter: Log	Mean peak liter: Geometric $\times 10^3$	Mean peak liter: Arithmetic $\times 10^3$	Rate of accumulation k: First phase	Rate of accumulation k: Derived when > one phase	Length in days of: Induction period	Length in days of: Antibody rise to peak	Number of rabbits
A. First injection before irradiation and others after irradiation										
5	13	0.125	4.07 ± .08	11.75	13.91 ± 3.06	1.3 ± .07	0.9 ± .07	8.6 ± 0.6*	7.8 ± 0.5	9
6		0.125	Not tested							
7	18	10	4.30 ± .12	19.93	26.75 ± 5.63	1.3 ± .10	1.1 ± .11	5.7 ± 0.4*	7.0 ± 0.5*	10
B. All injections after irradiation										
5	14	0.125	3.11 ± .24+	1.29	2.98 ± 0.96+	0.9 ± .23*	0.6 ± .08*	11.3 ± 1.1*	7.5 ± 0.9	8
6‡	16	0.125	1.57 ± .21*	0.037	0.06 ± 0.02*	0.6	0.3	14.4	5.6	2 (4)
7	19	10	3.67 ± .14*	4.68	6.14 ± 1.88+	1.0 ± .17+	0.8 ± .09	7.6 ± 0.1*	8.8 ± 1.1	7
C. Red cells alone: no X rays										
5	15	0.125	3.96 ± .10	9.13	11.13 ± 2.54	1.4 ± .12	1.1 ± .20	3.4 ± 0.36	8.3 ± 2.10	8
6‡	17	0.125	3.74 ± .15	5.50	6.23 ± 1.32	2.1 ± .37	1.5 ± .47	3.6 ± 0.31	5.9 ± 1.33	6
7	20	10	4.13 ± .05	13.49	15.51 ± 1.68	1.6 ± .21	0.8 ± .13	2.5 ± 0.13	10.5 ± 1.11	22

† See first footnote in table 1.
‡ The 10 injections in experiment 6 were made during 3½ days.

gen injections was carried out with individual doses of 0.125 ml and 10 ml of 1% cells/kg rabbit. Nine or 10 injections were given within a period of 15 to 17 days in experiments 5 and 7. Typical injection schedules and the main features encountered in these experiments are shown in figure 3 by three typical

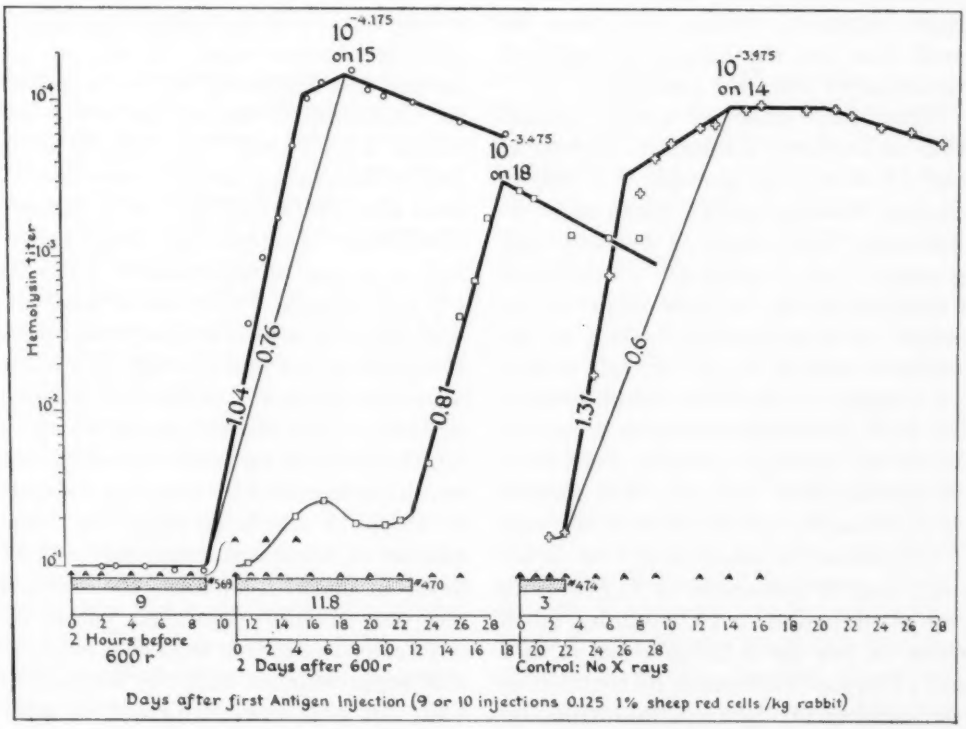

FIG. 3.—Representative examples of series 13, 14 and 15 in experiment 5 showing the hemolysin curve from 3 rabbits given multiple injections of 0.125 ml 1% cells. The time of the first antigen injection is noted with respect to irradiation and the antigen injections are indicated by arrows.

rabbits from series 13, 14 and 15 of experiment 5.

Enhancement of antibody formation could not be studied by giving the entire series of injections before X irradiation because by the 15th day serum antibody is rising very slowly or falling. Under such conditions we have found no demonstrable effect of heavy X irradiation and, even if a retardation of antibody formation occcurred, it could not be detected because the rate of formation is so slow (see discussion in our earlier paper, 1950b).

We did, however, test the effect of injecting the first dose 1 to 4 hours before irradiation with 500 or 600 r. Under such conditions, there was a significant lengthening of the induction period (cf. series 13 with series 15 and series 18 with series 20). Otherwise, there was little indication of any effect on the antibody response. Neither the mean log peak titer nor the values of k differed significantly from the controls.

The minor effect of heavy irradiation on antibody formation in series 13 and 18 is striking because 9 of the 10 antigen injections were made after irradiation when there is marked suppression of antibody formation. Furthermore, the animals might be expected to form antibody equal to the amounts formed by (1) animals receiving a single antigen dose before irradiation plus (2) animals receiving the entire series of multiple antigen injections after irradiation. Actually, they formed more. Thus, for the 10 ml dose, the sum of the geometric mean peak titer in series 7 and 19 amounted to 15,880 units /ml as compared to 19,930 units/ml in series 18. For the 0.125 ml dose, series 1 and 14 formed 6790 units/ml together as compared to 11,750 units/ml formed by series 13.

When rabbits were given the entire 15- to 17-day course of injections after irradiation with 500 or 600 r, there was a marked inhibition of antibody formation with both antigen doses (series 14 and 19) as compared to the controls. Nevertheless, considerable amounts of antibody were formed which were of the same order of magnitude as nonirradiated controls receiving a single injection. Thus, series 14 receiving 9 or 10 injections of 0.125 ml 1% cells/kg rabbit after irradiation (table 2) attained a geometric mean of 1290 units/ml as compared to 1780 units/ml for nonirradiated series 3 receiving only one injection of the same size. The same comparison for the antigen dose of 10 ml of 1% red cells/kg rabbit (series 19 vs 9) showed a geometric mean of 4680 units /ml for serial injections after irradiation and 2700 units/ml for a single injection in nonirradiated animals.

That the formation of antibody following serial injections beginning 2 days after irradiation might be due to recovery was suggested by the fact that we encountered one rabbit which attained a high peak titer (10,000 units /ml) following an antigen injection 10 days after 700 r (see fig. 2 of Taliaferro et al, 1952). We, therefore, tested the effect of giving 10 injections of 0.125 ml 1% red cells/kg within an 80 hour period. The first injection was made 2 days after irradiation and the last was made before there was any evidence of marked recovery. Unirradiated control series 17 attained a mean log peak titer which did not differ significantly from the controls in series 15 which received the same number of injections extending over 17 days. In marked contrast, the animals receiving multiple injections within $5\frac{1}{3}$ days after irradiation showed a remarkable suppression of antibody formation. Four out of 6 animals formed no measurable antibody. The geometric mean titer was only 37 units/ml ($P=0.001$), which was approximately the same

TABLE 3.—*Primary hemolysin response† following one injection of 1.25 ml 1% sheep red cells per kg rabbit (A) 1 to 4 hours before or (B) 2 days after irradiation along with (C) controls. The irradiation varied from 50 r to 700 r total body.*

Exp. and series		X rays	Mean peak titer: Log	Mean peak titer: Geometric $\times 10^3$	Mean peak titer: Arithmetic $\times 10^3$	Rate of accumulation k: First phase	Rate of accumulation k: Derived when > one phase	Length in days of: Induction period	Length in days of: Antibody rise to peak	Number of rabbits
					A. One injection 1 to 4 hours before X rays					
8	21	10 r	4.01 ± .13*	10.23	13.46 ± 2.59*	2.0 ± .28*	1.2 ± .11	2.2 ± 0.03*	5.5 ± 0.2*	10
9	22	25 r	3.78 ± .13*	6.03	7.55 ± 0.98*	1.7 ± .24+	1.4 ± .24	2.7 ± 0.1	5.4 ± 1.3	10
10	24	50 r	3.59 ± .13	3.89	7.54 ± 1.72+	2.0 ± .17*	1.3 ± .18	3.0 ± 0.1	5.4 ± 0.1*	20
11	26	100 r	3.49 ± .13	3.09	4.15 ± 1.01	1.8 ± .32+		2.4 ± 0.1+	3.9 ± 0.5	10
12	28	200 r	3.51 ± .08	3.24	3.85 ± 0.76	1.6 ± .21	1.0 ± .12	2.7 ± 0.2	5.8 ± 0.5*	11
13	30	300 r	3.81 ± .09*	6.55	7.73 ± 1.74*	1.1 ± .06	0.8 ± .07+	3.7 ± 0.3	7.7 ± 0.7*	7
14	32	400 r	3.62 ± .07*	4.17	4.80 ± 0.79	0.8 ± .08*	0.6 ± .06*	4.3 ± 0.2*	8.9 ± 0.7*	10
15	34	500 r	4.00 ± .09*	10.00	11.31 ± 2.19*	1.0 ± .14*	0.7 ± .07*	5.5 ± 0.8*	10.7 ± 0.8*	7
16	36	600 r	3.80 ± .10*	6.31	9.10 ± 3.12*	0.9 ± .06+	0.7 ± .05+	5.9 ± 0.3*	9.0 ± 0.6*	13
17	38	700 r	3.84 ± .14*	6.92	9.85 ± 2.11*	1.1 ± .13	0.7 ± .09*	7.5 ± 0.6*	9.9 ± 1.6*	11
					B. One injection 2 days after X rays					
8		10 r	Not done							
9	23	25 r	3.66 ± .14	4.57	5.94 ± 1.80	1.9 ± .24+	1.6 ± .33*	3.1 ± 0.3	4.8 ± 1.0	6
10	25	50 r	3.62 ± .10	4.17	4.90 ± 1.06	1.3 ± .09	1.0 ± .09	2.9 ± 0.3	6.5 ± 1.4*	8
11	27	100 r	3.51 ± .13	3.24	4.10 ± 0.94	1.3 ± .13	1.2 ± .18	4.7 ± 0.4	6.1 ± 1.4*	8
12	29	200 r	2.51 ± .11*	0.32	0.55 ± 0.11*	0.8 ± .14*	0.6 ± .14*	5.4 ± 0.5*	7.4 ± 1.9*	9 (1)
13	31	300 r	2.48 ± .24*	0.30	0.56 ± 0.11*	1.0 ± .06*		7.7 ± 1.1*	3.5 ± 0.2*	8 (1)
14	33	400 r	1.77 ± .13*	0.06	0.09 ± 0.01+	0.3 ± .07*	0.2 ± .07*	10.7 ± 2.5*	11.8 ± 3.0*	5 (3)
15	35‡	500 r	1.68 ± .11*	0.05	0.05 ± 0.01*	0.3 ± .05*	0.2 ± .05*	14.2 ± 2.0*	9.7 ± 2.6*	5 (2)
16	37‡	600 r	1.94 ± .18*	0.09	0.16 ± 0.01*	0.8 ± .15*	0.4 ± .05*	11.5 ± 0.9*	7.3 ± 1.9*	7 (3)
17	39	700 r	2.07 ± .17*	0.12	0.39 ± 0.16*	0.5 ± .03+	0.4 ± .01*	7.7 ± 1.0*	7.2 ± 1.9*	8 (4)
					C. Controls: no X rays					
8, 17	40§	0	3.40 ± .04	2.52	3.60 ± 0.37	1.3 ± .05	1.2 ± .05	3.2 ± 0.11	4.3 ± 0.16	71

† See first footnote in table 1.
‡ Antigen given either 1 or 2 days after X rays in series 35 and 37.
§ Same as series 6 in experiment 2.

amount of antibody formed in irradiated animals after a single antigen dose (series 2). For the two rabbits showing a rise in titer, the derived k of only 0.3 and the long induction period of 14.4 days suggest that the immune process was considerably retarded.

The relation of irradiation dosage to X-ray modification of the antibody response. —Table 3 shows the data for antibody formation in rabbits receiving 1.25 ml of 1% sheep red cells/kg before or after total body irradiation ranging from 10 r to 700 r.

When antigen was given 1 to 4 hours before irradiation, log peak titer was significantly higher in the low range of 10 and 25 r and in the high range of 300 to 700 r. The enhancement with 300 r and above was similar to the "stimulation" described by us and Janssen(1952) and was associated with a decrease in the rate of the immune process. Thus, the induction period was significantly longer ($P \leqq 0.001$) with all doses except the 300 r dose, and the derived value of k was smaller in all cases. In addition, the duration of the antibody rise to peak titer was significantly lengthened, as it would have to be with a higher peak titer and a smaller value of k.

The enhancement of antibody formation obtained with the lowest two X-ray doses was different from the preceding enhancement in that no retardation of the immune process occurred. With 25 r, the higher mean log peak titer was associated with no significant change in the derived value of k or the length of either the induction period or the rise to peak titer. With 10 r, however, the induction period was significantly shortened. It should also be noted that the value of k during the first phase was significantly increased for all doses from 10 to 100 r, inclusive. This increase may be associated with a stimulation of splenic activity since it occurred during

the first part of the antibody rise when the spleen is most active (see our earlier paper, 1951b).

When antigen was given 2 days after irradiation, all series receiving from 200 to 700 r inclusive showed a marked and highly significant ($P \leqq 0.001$) reduction in mean log peak titer as compared to the controls. The greatest reduction was shown by the 7 rabbits in series 35, which received 500 r and which attained a geometrical mean of only 50 units/ml while control series 40 reached 2520 units/ml. Associated with the reduction in peak titer, all measures of the rate of the immune process showed a statistically significant retardation in animals which formed measurable amounts of antibody. The probabilities for most of these differences were 0.001 or less and only two were larger than 0.01 (table 3). The probabilities for the differences of k and of the initial rise to peak between series 31 and 40 were only 0.05.

DISCUSSION

The foregoing experimental results and their statistical analysis leave little doubt that antibody formation may be significantly enhanced when antigen is given 1 to 4 hours before irradiation and significantly suppressed when antigen is given 2 days after irradiation. Similarly, these responses were significantly modified by the dosage of X rays and by the amount of single antigen injections or by the repetition of antigen injections. The question arises as to whether there is a significant correlation between antigen dosage and antibody response and whether an approximate dosage-response curve can be constructed for the X-ray modifications of antibody formation.

Most investigators have failed to find any marked correlation between the amount of antigen injected and the amount of antibody formed in normal animals. Earlier, we (1950) found no significant difference in peak titer of rabbits subsequent to a single injection of 0.125, 1.25 or 10 ml of 1% cells. After the largest amount of antigen, however, the rise of antibody to peak titer was more frequently diphasic, i.e., the initial high rate of accumulation in the serum was followed by a second smaller rate of accumulation. With the same peak titer and the same average rate (derived k), there would obviously have to be a higher rate of formation for the initial rise of the immune process. These findings have been extended in the present work. Within this antigen dose range (series 3, 6 and 9), the mean log peak titer was still not significantly different for any of the three amounts, but was significantly larger following the 250 ml dose (series 12) than after the 0.125 ml ($P = 0.01$) or 1.25 ml ($P = 0.05$) dose. In addition, the graph of mean log peak titers plotted against log antigen dose indicated a linear relationship.

The regression of log peak titer (Y) on the log of antigen dose (X) for 146 unirradiated control rabbits in experiments 1 through 4 is shown in figure 4. Although the relationship is linear, it is at once apparent that an increase in the log antigen dose resulted in very little increase in log titer. Nevertheless, the slope of the regression line of log peak titer on log antigen dose ($b = 0.095 \pm 0.031$) and the coefficient of correlation ($r = 0.254$) showed a probability of < 0.01. As the antigen dose was increased, there was no consistent trend in either the average rate of antibody rise (derived k) or the length of the antibody rise. The first part of the antibody response was accelerated, however, as indicated by a decrease in the length of the induction period and an increase in the initial rate of accumulation (k).

In marked contrast to the compara-

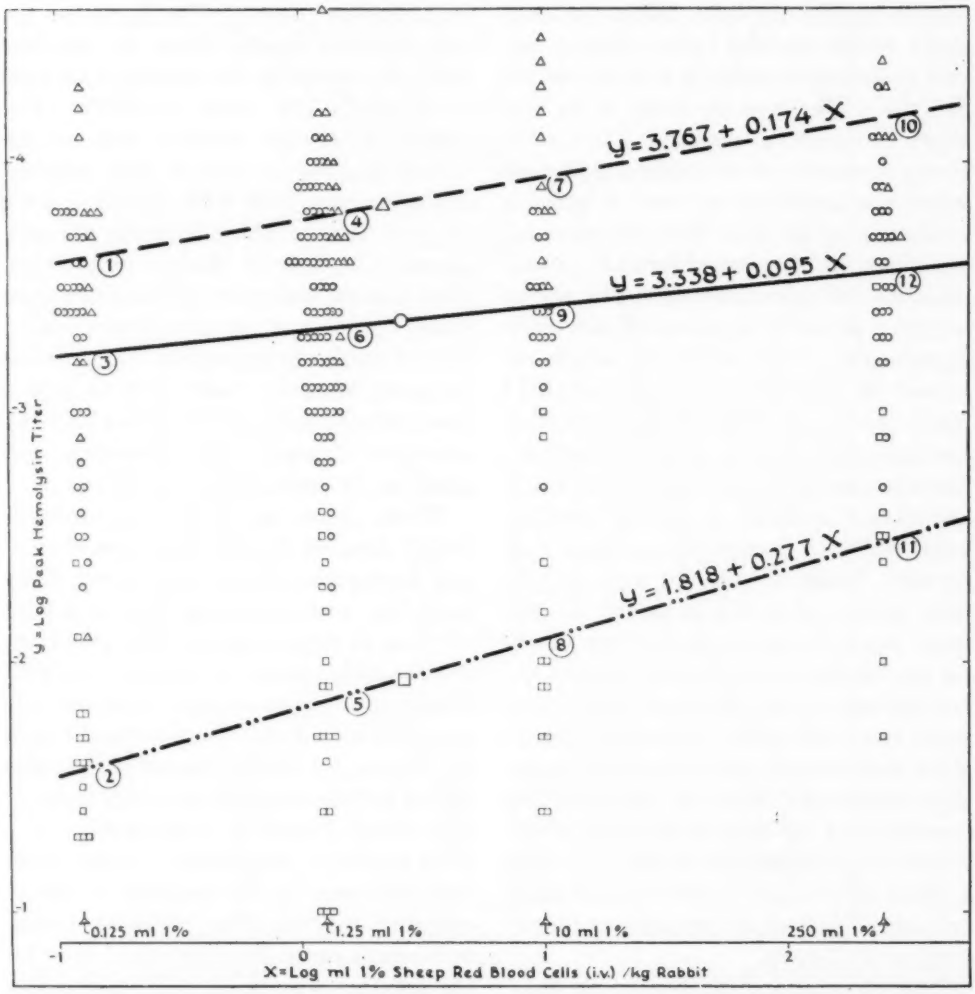
y = 3.767 + 0.174 X
y = 3.338 + 0.095 X
y = 1.818 + 0.277 X
y = Log Peak Hemolysin Titer
0.125 ml 1%
1.25 ml 1%
10 ml 1%
250 ml 1%
X=Log ml 1% Sheep Red Blood Cells (i.v.) /kg Rabbit

FIG. 4.—Regression of log peak hemolysin titer (y) on log antigen dose (x) in experiments 1 through 4. The series numbers are circled. Data points for individual rabbits are indicated by triangles for those receiving antigen 1–4 hours before irradiation (enhancement of peak titer); by circles for unirradiated controls; and by squares for those receiving antigen 2 days after irradiation (suppression of peak titer). The Y and X means are indicated on the appropriate regression line by large symbols of the same type.

tive ineffectiveness of increasing the size of a single antigen dose over a 2000-fold range, dividing a given dose into a series of smaller injections was remarkably effective in increasing peak titer. Thus, giving 10 injections of 0.125 ml/kg resulted in a log peak titer of 3.96 (series 15 in table 2) as contrasted with 3.4 (series 6, table 1) when the same total amount was given in one injection. This difference ($P<0.001$) was markedly significant and represented a difference between the geometric means of 6610 units/ml. The spacing and probably the size of the antigen dose must be of importance in this increased stimulation. We (1952) have previously shown that the spleen probably largely stops forming antibody at about peak titer with 1 dose of 1.25 ml of 1%/kg, but will form

approximately the same amount of antibody within normal limits when a second injection of antigen is given on the 7th day. We have no data as to how short the interval may be. That additional amounts of antibody are formed when the intervals are only 8 hours is indicated by the fact that the mean log peak titer (3.74 units) of series 17, which received 10 injections of 0.125 ml/kg within a period of $3\frac{1}{3}$ days, did not differ significantly from series 15 which received 10 injections over a period of 17 days. On the other hand, repeated antigen injections lead in time to a truly refractory period during which little if any additional antibody is formed and serum antibody decays faster than it is formed. Thus, as may be seen by the data from rabbit 474 in figure 3, antibody began to accumulate more slowly on the 7th day and virtually stopped accumulating on the 14th day while injections were still being continued. Somewhat similarly, at least a third of the antigen injections failed to stimulate the formation of additional amounts of antibody in the rabbits of series 15 in table 2 which were injected for 15 to 17 days because peak titer was reached on an average of 11.7 days.

The effect of antigen dosage on antibody formation was somewhat greater in the irradiated animals. The regression lines are shown in figure 4. When antigen was injected before irradiation, $b = 0.174 \pm 0.059$ and $r = 0.417$ ($P < 0.01$). Data in table 1 suggest that the relationship ceased to be linear above the dose of 10 ml of 1% cells as the response was practically the same after the two highest doses. Without the highest dose, the constants become $b = 0.251 \pm 0.099$ and $r = 0.39$ ($P < 0.01$).

The regression was highest when antigen was given 2 days after irradiation: $b = 0.277 \pm 0.069$ and $r = 0.535$ ($P < 0.001$). Data in table 1 indicate that the relationship ceased to be linear for the two smallest doses. When the smallest dose was omitted, the constants become $b = 0.341 \pm 0.107$ and $r = 0.531$ ($P < 0.001$). It is thus evident that an increase of a log in antigen dose resulted in an increase from 0.28 to 0.34 of a log in peak titer in animals under the suppressive action of heavy irradiation. This finding indicates that large antigen doses "protected" against X-ray inhibition of antibody formation. The relative increase, however, took place at such a low level of antibody formation that the absolute changes were probably too small to be biologically significant.

There seems no doubt that animals which received 9 or 10 injections of antigen beginning 2 days after heavy X irradiation and extending over a period of 15 to 17 days attained peak titers approximating those in normal controls receiving a single antigen injection (cf. series 14 with 3 and 6 and series 19 with 9). Earlier, we (1953) suggested that this might be the result of recovery from X rays which begins in some rabbits at 7 days and/or a summation of the small responses seen in the majority of the irradiated rabbits. The recovery factor now seems more important and may be the sole factor since antibody formation was as markedly inhibited in rabbits immunized within $5\frac{1}{3}$ days after irradiation as in those receiving a single antigen injection (cf. series 16 with 2 and 5). Furthermore, the antibody-forming capacity appears blocked to the extent that repeated antigen injections were no more effective than a single one.

Less clearly understood is the result that serially injected animals receiving the first injection 1 to 4 hours before and the remainder after irradiation formed unexpectedly large amounts of antibody. In both groups receiving multiple doses of 0.125 and 10 ml 1% cells/kg (series 13 and 18), the geometric mean

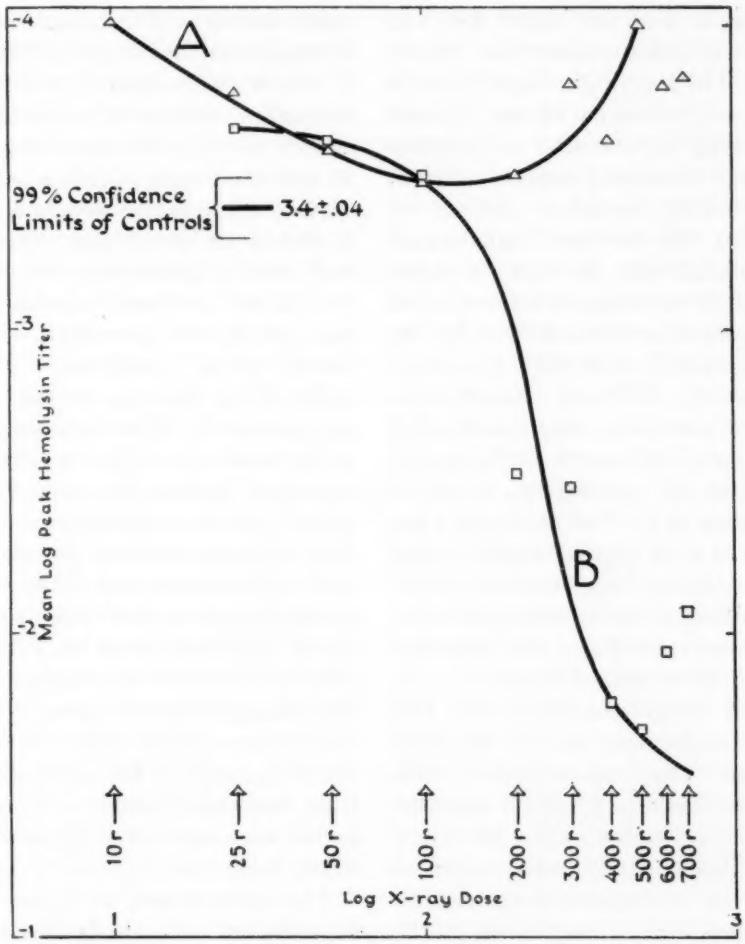

FIG. 5.—Dosage-response curves relating log peak hemolysin titer to log dose of X rays in experiments 8 through 17 (controls = series 40): 1.25 ml of 1% red cells given (A) 1 to 4 hours before irradiation, and (B) 2 days after irradiation. Responses for 600 r and 700 r were disregarded in the visual fitting of both curves (see text).

peak titers were larger than the sum of the mean of (1) rabbits receiving one injection before irradiation and (2) rabbits receiving 10 injections starting 2 days after irradiation. More work is needed on this problem. That the injection before irradiation permitted a series of anamnestic responses later is not tenable because we (1954 and also with Janssen, 1952) have shown that the anamnestic response is as susceptible to X irradiation as the initial response.

Varying amounts of irradiation led to two types of increase in log peak titer when antigen was injected 1 to 4 hours beforehand. The first enhancement with a pronounced retardation of the immune process occurred to a statistically significant degree with 300 r and higher doses of X rays. The second one, without retardation and at times with some evidence of an increased rate of the immune process, occurred to a statistically significant degree at 10 and 25 r. A dosage-response curve made by plotting mean log peak titer against log

X-ray dose is shown in figure 5A. The two types of enhancement are clearly indicated. The seemingly single curve is probably a combination of two sigmoid curves, which have similar asymptotes in the range of normal antibody formation (50 to 200 r inclusive) and are incomplete at the low and high dosage ranges, respectively. In fitting a curve in the high X-ray range, we disregarded the data points for 600 and 700 r for the reasons discussed more fully in considering figure 5B. Without a more complete curve, there is no way of indicating a median effective dose. A probit analysis was made by plotting the probit of enhancement, defined as attaining a log peak titer of 4 log units or more against log antigen dosage, but was unsatisfactory because most of the data points occurred below probit 5 and the curve was very flat (high standard error).

It seems improbable that the two types of enhancement involve the same mechanism. The enhancement with large X-ray doses is hardly a stimulation because it is associated with injury to the antibody-forming mechanism as evidenced by a retardation of the immune process, i.e., a lengthening of the induction period, a lowering of the rate of rise (k) of serum antibody, and a lengthening of the rise to peak titer. (See discussion in our later paper, 1954).

The enhancement of peak titer following small doses of X rays without retardation of the immune response may be a true stimulation and was entirely unexpected. We are skeptical that such small X-ray exposures can produce such significant increases in antibody formation. It is entirely possible that they arose from unknown factors introduced into series 21 and 22. Against this is the fact that the controls showed no unusual antibody responses. More work is needed here.

In the clinical literature, there are many accounts of the curative action of X irradiation on certain infections, some of which are supposed to be mediated through an enhanced production or liberation of antibody. Assuming that such an increase occurs at times (many of the studies are actually poorly controlled), it should be noted that irradiation in such work is given after the infection is established and hence during a more or less continuous liberation of antigen. Under such conditions, stimulation might occur because of the release of antigen shortly before irradiation. In our work, however, antigen injected *after* irradiation during the time of marked injury did not stimulate the formation of much antibody (series 16, table 2). It is also noteworthy that we did not obtain unequivocable evidence of stimulation by small doses of X rays given after the injection of antigen (series 23). The dosage-response curve in figure 5B for the lower doses, 25 to 100 r, is above the 99% range of the unirradiated controls, but neither singly nor grouped together were peak titers significantly different from the controls.

The marked and, at times, complete suppression of antibody formation which occurred when antigen was injected 2 days after irradiation is sharply limited both as to the part of the immune response involved (see our paper following this, 1954, Dixon et al, 1952, and Taliaferro et al, 1952) and as to the minimum X-ray dose necessary to produce it to a significant degree. The experimental work described in the present paper (table 3) indicates that all doses of X rays ranging from 200 r to 700 r, inclusive, produced a statistically significant reduction in log peak titer, whereas all doses of 100 r and lower failed to do so.

In attempting to construct dosage-response curves for the X-ray suppression of antibody curves, we used both quan-

titative curves and the all or none probit method.* The latter was suggested by the arithmetic peak titers shown in figure 2 of our earlier paper with Janssen (1952) and by the possibility that stimulation (when antigen is given before irradiation) and recovery of the ability to respond within the normal range (when antigen is given after irradiation) may be a semi-quantal phenomenon.

The dosage-response curve for animals receiving antigen 2 days after irradiation is shown in figure 5B. It is typically sigmoid with only the response at 600 and 700 r markedly out of line. The latter results may be associated with the fact that many deaths occurred with 600 r and especially with 700 r and with the further fact that death may have been proportionately higher in rabbits in which antibody formation would have been completely suppressed. In this connection, it should be pointed out that Smith and Gump (1954) found that the capacity to form hemolysins and precipitins before midlethal irradiation was not correlated with survival following midlethal irradiation. This does not necessarily bear on the point at hand because their data do not actually correlate survival with the ability of X-rays to give complete as contrasted with partial inhibition. Since we were unable to resolve this dilemma, we disregarded the 600 and 700 r doses in fitting the curve. If the curve is bent on itself, the midpoint of the linear portion of the curve is at about log X-ray dose 2.38 or 240 r. This figure compares remarkably well with the use of a log peak titer of less than 2.2 for suppression when plotting log X-ray dose against the probit transformation of percent suppression. This value for suppression was used because it was always exceeded in controls, i.e., in all of the 182 nonirradiated rabbits. The probit analysis indicates an ED_{50} approximating 250 ± 39 r. In this analysis, the data for 600 and 700 r were also disregarded because they gave only 60 and 67% suppression, respectively, whereas 500 r gave 100% suppression.

The probit method might conceivably be used with complete antibody suppression as a criterion, i.e., antibody never rising above the preimmunization titer. It was not possible in the present work, however, because the percentage of rabbits showing complete suppression decreased in the high X-ray range (last column in table 3). This again may be a reflection of the disproportionate number of deaths among the rabbits irradiated with 600 and especially 700 r.

CONCLUSIONS

The following conclusions on hemolysin formation are based on photometric determinations of 50% hemolytic units in rabbits immunized intravenously with whole sheep red cells. The amount of antibody formed was evaluated in terms of log peak serum titer and the rate of the immune process was measured in terms of the length of the induction period and values for k (rate of rise in the first order equation describing the rise of antibody rise of antibody in the serum) either for the first phase of the antibody rise or an average k for biphasic rises.

1. Three effects were noted when a sin-

* In considering some of our data by the probit method in the discussion, we have made graphic estimations of the ED_{50} and its standard error by using the logarithmic-probit graph paper and the graphic methods described by Miller and Tainter (1944). Where o or n reactors were found among n animals, we have considered them as $\frac{0.25}{n}\%$ and $\frac{n-0.25}{n}\%$, respectively, as suggested by Bartlett (1937).

gle antigen dose was held constant and the total body X-ray dose was varied from 10 r to 700 r.

(a) When antigen was given 2 days after adequate irradiation, antibody formation was totally suppressed in some animals and markedly suppressed in others. In those forming measurable amounts of antibody, the rate of the immune process was markedly decreased. Statistically valid suppression of antibody took place with 200 r but not with 100 r. A plot of mean log peak titer against log X-ray dose gave a sigmoid curve and indicated a median suppression at about 240 r. A probit analysis based on the percent of animals attaining log peak titers of 2.2 or less (a range under the normal controls) indicated an ED_{50} of 250 ± 39 r X-rays.

(b) When antigen was administered 1 to 4 hours before irradiation, two types of enhancement of antibody formation were found. With large doses of X rays (300 r to 700 r) an enhancement of mean peak titer was associated with a marked retardation in the immune process.

(c) With small doses of X rays (10 r and 25 r but not with 50 r), the enhancement occurred with no slowing of the immune process. Neither type of enhancement will explain the reported curative action of X rays on infections because any enhancing action from antigen absorbed before irradiation would be more than offset by the suppression of antibody from antigen liberated after irradiation.

2. The size of a single antigen injection extending over a 2000-fold range gave a higher correlation with peak titer in irradiated than in control rabbits, as shown by the following:

(a) In unirradiated controls, the regression coefficient of log peak titer on log antigen dose was 0.095 ± 0.031 and the coefficient of correlation was 0.254.

(b) In the enhancement of peak titer when antigen was given before 500 or 600 r X irradiation, the regression of log peak titer on log antigen dose was 0.174 ± 0.059 and the coefficient of correlation was 0.417.

c. In the suppression of antibody formation when antigen was given 2 days after the same amount of irradiation, the regression of log peak titer on log antigen dose was 0.277 ± 0.069 and the coefficient of correlation was 0.535. As a result of the last relationship, it follows that increasing the dose of antigen given after irradiation resulted in highly significant increases in antibody formation, but the amounts were so small that they are probably biologically insignificant.

3. When repeated doses of antigen were given in a 17-day period beginning 1 to 4 hours before 500 or 600 r irradiation, no enhancement of peak titer occurred as compared with unirradiated controls, but the immune process was retarded to the extent that the induction period was significantly lengthened.

4. When the same injection schedule was started 2 days after the same amount of X rays as in 3, the mean peak titer was almost as high as in unirradiated controls receiving a single antigen injection. When, however, the same number of injections were given within a $5\frac{1}{3}$ day period after irradiation, the mean peak titer was no higher than after a single antigen injection given 2 days after irradiation (maximum suppression). It follows, therefore, that antibody formation after irradiation is due chiefly to the recovery of animals from irradiation and not to the summation of small antibody responses which occur in animals receiving single injections.

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FURTHER STUDIES ON THE RADIOSENSITIVE STAGES IN HEMOLYSIN FORMATION

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Severe total body irradiation with hard X rays has been found to affect antibody formation in three more or less distinct ways depending upon the time of irradiation in relation to the injection of antigen: (1) marked and at times complete suppression of antibody formation which only occurs when animals are irradiated before receiving antigen (Benjamin and Sluka, 1908; review in W. H. and L. G. Taliaferro, 1951; Kohn, 1951; Dixon et al, 1952; Taliaferro et al, 1952; and others), (2) a general retardation of the immune process with no lessening of the amount of antibody formed when animals are irradiated shortly before receiving antigen or afterwards during active antibody formation (Kohn, 1951; and Taliaferro et al, 1952) and (3) enhancement of antibody formation superimposed upon a general slowing of the rate of the immune process when irradiation is administered shortly after the antigen (Taliaferro et al, 1952).

The present work is an attempt to study more carefully the development and early recovery from the injury which results in maximum suppression of antibody formation and to characterize further the various radiosensitive periods. In addition, a restudy of the radiosensitivity of the anamnestic response was made.

MATERIALS AND METHODS

The same materials and methods were used as described in preceding papers (notably 1950, 1952 and 1954). Only a few salient points will be noted.

Sheep red cells were used exclusively for all immunizations and titrations. They were always standardized photometrically, but the amount for injection is usually expressed for convenience in terms of a 1% suspension. In the first 16 experiments, we used 0.125 ml of a 1% suspension (approximately 2×10^7) of red cells/kg rabbit. This is $\frac{1}{80}$th the amount given in our earlier work on irradiation (Taliaferro et al, 1952). This smaller amount was used to obtain a maximal suppression of antibody formation by X irradiation (see our previous paper in 1954). In the study of the radiosensitivity of the anamnestic response, we used 10 ml of a 1% suspension which is 8 times larger than the amount injected in our earlier work. This was done to lessen the X-ray inhibition of antibody formation. All injections were administered intravenously and per kg rabbit.

All titers are based on photometric determinations of hemolysis and are expressed in 50% hemolytic units either as (1) the amount of serum necessary to produce 50% hemolysis of 2 ml of 1% sheep red cells in the presence of 4 50% units of complement (5 ml total per test) or (2) as the number of such units per ml of serum.

Antibody formation is characterized by the following measurements as described in our preceding paper (1954): log peak titer as a measure of the amount of antibody formed, and, as measures of the rate of the immune process, the induction period in days, the rate (k) of antibody rise in the first order equation describing the initial rise of serum antibody or the derived rate

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for the entire initial rise when the latter consisted of two rates, and the length of antibody rise to peak titer. All of the measurements were taken from smoothed semilogarithmic plots of log antibody against time. When irradiated animals showed no rise above their preimmunization titer they were assigned their preimmunization titer. Adjusted peak titers and rates for the anamnestic response were calculated in accord with our previous practice (1952 and with Janssen, 1952).

Total body irradiation was used throughout and was administered by two machines located either in the Department of Anatomy or in the Argonne Research Hospital of the University of Chicago. The constants may be found in our previous paper (1954).

Rabbits were supplied either by a local dealer or by Mr. Harold Swift of Lakeside, Michigan, and consisted of approximately equal numbers of males and females.

EXPERIMENTAL RESULTS

Irradiation during the initial antibody response.—Our study of the time relationship of antigen injection and X irradiation involved 106 irradiated animals and 31 unirradiated controls. Series 4, 9, 10 and control series 16 were incorporated in our previous paper (1954) as the experiments in both papers were carried out conjointly.

Fifteen series containing from 4 to 12 rabbits were injected with 0.125 ml of 1% red cells/kg at various intervals before 500 r irradiation, i.e., 4, 2 and 1 day, 2 and 0.2 hours, and after 500 r irradiation, i.e., ½, 1 and 4 hours, 1 and 2 days and 1, 2, 3, 4 and 8 weeks. Each batch of rabbits was accompanied by two or more similarly injected but not irradiated controls. The dose of 500r X rays was used because this dose gave the greatest suppression of antibody formation and antibody enhancement, without killing many of the rabbits (see the dosage-response curve and discussion in our paper of 1954). Table 1 contains data on the hemolysin response in these series, along with standard errors and the statistical significance of differences between the 15 irradiated series and control series 16.

TABLE 1.—*Experiment 1: primary hemolysin response† following one intravenous injection of 0.125 ml of 1% sheep red cells per kg rabbit from 4 days before to 8 weeks after 500 r total body irradiation along with controls.*

Series	Time of red cell injection	Mean peak titer			Rate of accumulation k		Length in days of:		Number of rabbits
		Log	Geometric $\times 10^3$	Arithmetic $\times 10^3$	First phase	Derived when > one phase	Induction period	Antibody rise to peak	
				Red cells before 500 r					
1	4 days	2.76 ± .35	5.75	1.31 ± 0.48	0.7 ± .16*	0.4 ± .04*	4.1 ± 0.6	9.3 ± 2.6*	6
2	2 days	3.07 ± .31	1.17	2.60 ± 1.26	0.7 ± .22*	0.4 ± .11+	7.2 ± 1.6*	9.5 ± 2.3+	5
3	1 day	3.23 ± .50	1.70	1.72 ± 0.25	0.9 ± .16	0.7 ± .22	6.0 ± 0.2*	9.3 ± 3.5*	4
4	2 hours	3.74 ± .14+	5.50	8.08 ± 1.87*	0.9 ± .08	0.6 ± .05+	7.5 ± 0.7*	10.5 ± 0.8*	10
5	0.2 hours	3.09 ± .23	1.23	2.76 ± 1.24	1.3 ± .16	0.6 ± .11*	9.7 ± 0.6*	8.8 ± 0.8*	8 (1)
				Red cells after 500 r					
6	0.5 hours	3.29 ± .16	1.95	2.94 ± 0.97	1.1 ± .08	0.8 ± .14	7.7 ± 0.5*	7.2 ± 1.4	6
7	1 hour	2.96 ± .12	0.91	1.44 ± 0.50	0.9 ± .12	0.8 ± .11	9.5 ± 0.6*	5.3 ± 0.5	11
8	4 hours	2.63 ± .14+	0.43	0.67 ± 0.24+	0.9 ± .11	0.7 ± .11	9.3 ± 0.3*	7.8 ± 1.5	8
9	1 day	1.49 ± .10*	0.03	0.04 ± 0.01+	0.3		8.4	7.6	1 (9)
10	2 days	1.88 ± .12*	0.09	0.09 ± 0.02+	0.3 ± .03*	0.2 ± .02*	18.8 ± 2.5*	10.5 ± 1.9	6 (1)
11	1 week	2.54 ± .26+	0.35	0.59 ± 0.38	0.8 ± .12	0.4 ± .09*	8.8 ± 2.1*	11.4 ± 2.5*	4
12	2 weeks	2.33 ± .28+	0.21	0.38 ± 0.14*	0.6 ± .22*		12.0 ± 2.8*	5.7 ± 1.5	4
13	3 weeks	2.75 ± .42	0.56	1.57 ± 0.87	1.1 ± .29	0.9 ± .38	5.7 ± 0.4	6.0 ± 1.3	5
14	4 weeks	2.72 ± .21	0.52	1.01 ± 0.26	0.9 ± .09	0.9 ± .14	5.4 ± 0.7+	6.3 ± 2.4	7
15	8 weeks	3.41 ± .26	2.57	7.10 ± 2.53	1.1 ± .19	1.0 ± .20	3.7 ± 0.6	4.9 ± 0.7	12
				Red cells alone: no X rays					
16	Controls	3.25 ± .09	1.78	2.82 ± 0.39	1.1 ± .08	1.0 ± .08	3.6 ± 0.19	5.2 ± 0.47	31

† All values are means ± standard errors. Data from individual rabbits were obtained from fitted curves. Numbers in parenthesis give additional rabbits which showed no antibody response. Mean peak titers were calculated from the total sample; other values only from those rabbits which showed an antibody response. The significance of the difference between each irradiated group and the control group are indicated by the following probabilities:

* $P \leq 0.001$ + $P = 0.002$ to 0.01 * $P = 0.02$ to 0.05

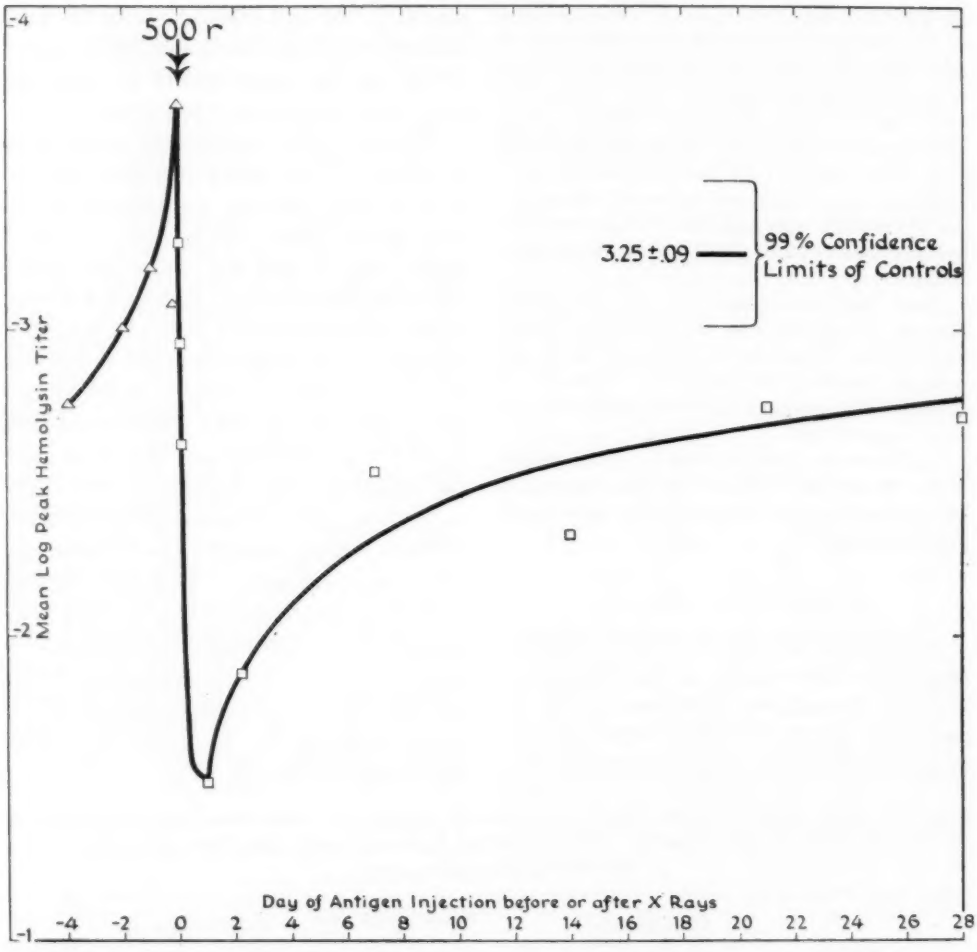
500 r
3.25 ± .09
99 % Confidence
Limits of Controls
Mean Log Peak Hemolysin Titer
-4
-3
-2
-1
Day of Antigen Injection before or after X Rays
-4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

FIG. 1.—Mean log peak hemolysin titer as related to the time of administering 0.125 ml of 1% red cells/kg rabbit before and after irradiation.

None of the first 5 series receiving antigen before irradiation had significantly lower mean peak titers than the controls, whereas series 4 receiving antigen 2 hours before irradiation reached a higher mean log titer ($P=0.01$). In these 5 series, the rate of the immune process was lowered as follows: (1) The length of the induction period was significantly increased in all but the −4 day group. In this group (series 1), the induction period could not have been affected because it had practically terminated at the time of irradiation. During the antibody rise, (2) the derived k was significantly decreased (except in the group of only 4 animals receiving antigen 1 day before irradiation) and (3) the antibody rise to peak titer was also significantly lengthened.

The mean peak titer was almost exactly that of the controls when antigen was injected 10 minutes before as well as ½ hour after irradiation. Beginning with the injection of antigen 1 hour after irradiation, the log peak titer decreased rapidly for 1 day as shown in figure 1. During this drop, the induction

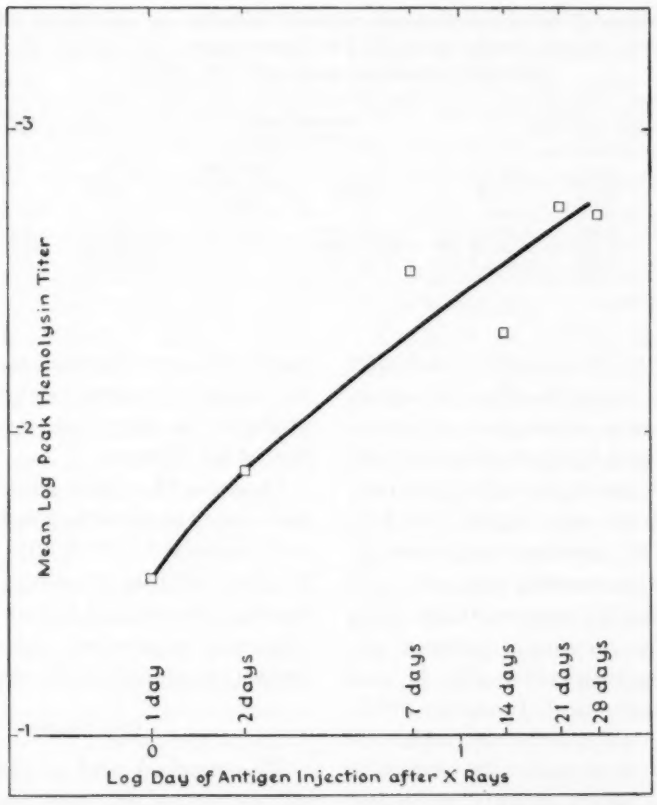

FIG. 2.—Recovery of antibody-forming power as indicated by the mean log peak hemolysin titer plotted against the log time of injecting 0.125 ml of 1% red cells/kg after irradiation.*

period was significantly longer, and the derived k was consistently lower although not significantly lower in series 6, 7 and 8. At one day after irradiation, 1 of 9 and, at 2 days, 6 of 7 rabbits showed a rise in titer. In the latter series, the rise was very slight (mean geometric peak titer of 90 units). Recovery at 1 week, as indicated by a series of 4 rabbits, was marked. Thereafter, it was less rapid and was not complete at 4 weeks. Thus, in series 14, 2 rabbits gave peak titers of 90 and 110 units, and the remaining 5 elicited responses within the normal range. These findings are essentially in accord with our previous data following the use of 10 times this amount of antigen. The data points from the 14th to the 28th day in figure 1 suggest a plateau, but they actually represent a rise because of the logarithmic curve shown in figure 2 and because recovery was complete by 8 weeks (table 1, series 15).*

Irradiation before or after a reinjection of red cells.—In experiment 2, the radiosensitivity of the anamnestic response was studied in 36 rabbits together with 18 unirradiated controls (table 2). One reinjection of 1 ml of 10% red cells/kg rabbit was given 1 to 2 hours before or 2 days after 500 r with a similar reinjection into unirradiated controls. The reinjection was made from 28 to 40 days after a first injection of sheep red cells.

The typical anamnestic response, as established by us earlier (1952), is shown

* Data for 8 weeks were added in proof.

TABLE 2.—*Experiment 2: secondary hemolysin response† following one intravenous injection of 1 ml of 10% sheep red cells per kg rabbit (17) 1 to 2 hours before or (18) 2 days after 500 r total body irradiation along with (19) controls.*

Series	Mean peak titer			Adjusted rate of accumulation (k)		Length in days of:		Number of Rabbits
	Log	Geometric $\times 10^2$	Arithmetic $\times 10^2$	First phase	Derived when >one phase	Induction period	Antibody rise to peak	
17	3.39 ± .41	2.46	8.53 ± 2.73	1.4 ± .21*	1.3 ± .22*	3.4 ± 0.2*	5.8 ± 0.8*	20(1)
18	1.76 ± .17*	.057	0.34 ± 0.20*	0.5 ± .20*		9.0 ± 3.2*	6.2 ± 1.8*	5(10)
19	3.43 ± .10	2.70	3.87 ± 0.59	2.0 ± .20		2.0 ± 0.13	2.7 ± 0.2	18

† See footnote in table 1.

by control series 19 in table 2. Peak titer was within the range of a first injection, but the induction period and initial rise were shorter, and the rate of accumulation consisted almost invariably of only one phase which was higher ($k = 2.0$). Series 17 and 18 corroborate the susceptibility of the anamnestic response to X rays as reported by us previously using one eighth the amount of antigen also given as a single injection (W. H. and L. G. Taliaferro and Janssen, 1952). Furthermore, all means in series 17 through 19 were statistically similar to corresponding series already published by us and Janssen (1952).

For example, in series 17, of 21 rabbits reinjected 1 to 2 hours before 500 r, one gave no response and, in the remaining 20 rabbits, the log peak titer varied from 2.1 to 4.6. This latter response, which was attained in two rabbits, may possibly represent an enhancement associated with the injection of antigen before irradiation. In the 20 rabbits which showed a rise in titer, the induction period and initial rise were lengthened and the rates decreased as compared to series 19. The probabilities for these conclusions are shown in table 2. The induction period was not lengthened as much in series 17 as in series 18 (3.4 vs 9.0 days). It was, however, not as much affected in the initial response (7.5 days in series 4 vs 18.8 days in series 10). Furthermore, it was 1.7 times the unirradiated anamnestic controls (series 19) as compared to 2.1 times for the initial response (series 4 vs 16). It probably is, therefore, appreciably retarded by X rays.

In series 18, which received antigen 2 days after radiation, the log peak titer was decreased ($P < 0.001$). In addition, in the 5 rabbits showing a rise, the induction period and initial rise were significantly lengthened and the rate of increase (k) significantly decreased.

DISCUSSION

The marked and at times complete suppression of antibody formation when antigen is injected after severe X irradiation was first described by Benjamin and Sluka (1908) and has been most studied. The sharp break in the ability to form antibody, as the time between irradiation and antigen injection is increased, is shown in figure 1. This represents the development of the X-ray injury which prevents antibody formation. Its presence was recognized by Kohn (1951) and Dixon et al (1952). The present work indicates that the drop in log peak antibody is almost linear with arithmetic time from the enhanced log peak titer at the 2-hour preirradiation time to 1 day postirradiation with the exception of the 12-minute preirradiation point.

Recovery of the power to form antibody was rapid during the first week and much slower thereafter. A plot of mean log peak antibody against log of

postirradiation time of antigen injection (fig. 2) indicates an approximately linear relationship. Recovery was complete at 8 weeks (table 1). In considering the dosage response curve of antibody on X-ray dose in our previous paper (1954), we made a probit analysis in which we defined suppression as the attainment of a log peak titer of 2.2 or less, which is a value below the range of all controls so far studied. A plot of probit of percent suppressed, as thus defined, against the log of time of antigen injection after irradiation, showed 90% suppression at one day with decreasing amounts at later times. The median time necessary for 50% recovery was 4.05 ± 1.6 days.

The radiosensitive period involved in the marked suppression of antibody must involve an early stage, although not necessarily the first stage, in antibody formation because antigen injected immediately before irradiation (table 1, series 5) allowed antibody synthesis to proceed in spite of X-ray injury. It is of short duration, but the fact that rabbits lose their power to form antibody progressively for a period of 24 hours after 500 r makes it impossible to sharply delimit the length of this radiosensitive period. Animals irradiated 1 hour previous to antigen injection attained a log peak titer somewhat lower (series 7) but not significantly different from the controls, whereas animals irradiated 4 hours before receiving antigen suffered a marked and statistically significant suppression of antibody formation. Therefore, for this degree of injury, there was a 1-hour latent period and a radiosensitive period of not more than 3 hours. There is nothing in the data to preclude a short or even almost instantaneous interval for the radiosensitive period giving this manifestation of injury.

Kohn (1951) suggested that this type of X-ray injury might be related to the radiosensitivity of the initial events involved in the fixation of the antigen by the cells. In our work with Janssen (1952), we suggested that it was probably due to the localization of the antigen in the antibody-forming sites and/or the early metabolism of the antigen. Dixon et al (1952) believed it involved the adaptation of globulin synthesis to the new antigen.

Fitch et al (1953) and Ingraham (1954) found no marked difference in the gross and Ingraham (personal communication) no difference in the intracellular distribution of antigen following heavy total body X irradiation as contrasted to unirradiated controls. Part of Ingraham's work involved the use of unheated sheep cell stromata which contain the hemolysinogenic antigens of whole sheep red cells. At present, however, there is no way of knowing whether these studies bear on the localization of the functional antigen in antibody-forming sites. The most obvious localization of antigen, such as that within the macrophages, is probably largely associated with removal and disposal of foreign material and such localized material may have nothing to do with antibody formation.

Harris et al (1954) found that when normal lymph node cells were incubated at 37 C in vitro with dysentery organisms or with a soluble antigen from them, washed and then injected into X-irradiated rabbits, antibodies appeared in the recipients within 4 days. Similarly, Jaroslow et al (1954) reported that boiled sheep cell stromata, after being mixed and incubated for 15 minutes at room temperature with splenic mince, were antigenic in rabbits which had received 400 r 1 day before.

The retardation of the immune process (lengthening of the induction period and lowering of the rate, k, of rise of serum antibody) without a significant

reduction in peak titer was studied by Kohn (1951) and especially by us with Janssen (1952). We pointed out that it probably occurs whenever irradiation is given after the antigen, provided antibody is being formed, but that it can probably only be detected when antibody is being formed at a high rate. It may be for this reason that we (1950b) were unable to obtain any evidence of X-ray effect on the post peak hemolysin decline. (Cf. Wissler et al, 1953.)

It seems probable from our earlier work with Janssen (1952) and the present study that the period between irradiation and any noticeable effect on the rate of the immune process is short although it may be as long as with the marked suppression of antibody. Thus, the data in table 1 indicate that irradiation at the end of the mean induction period (series 1) still permits a significant lowering of the rate (k) of the initial rise and the mean or derived k for the entire rise to peak titer. The radiosensitivity of the processes determining the length of the induction period is also striking. Thus, irradiation 2 days after antigen injection (table 1, series 2), when over half of the mean induction period of 3.6 days as shown by the controls had passed, resulted in a doubling of the period (7.2 days). In view of the studies of Green and Anker (1954) on the synthesis of antibodies by isotopic labeling methods, this finding suggests a change in the preliminary synthesis which takes place before release of antibody into the serum. If, as they believe possible, the preliminary synthesis is of an inactive precursor, which is made active at the time of release into the serum, it is interesting that the rate (derived k) of antibody rise is also lengthened.

The enhancement of peak antibody is localized in time (table 1) as it occurred when antigen was injected 2 hours before irradiation but not at either 1 day before or 12 minutes before. We did not attempt to ascertain the limits of this period more exactly because, in our earlier work with Janssen (1952), we found that an occasional animal irradiated from 2 days after to 6 hours before receiving antigen attained a peak titer above the range of the normals although most enhancement was in animals irradiated from 12 minutes to 3 hours after antigen injection. Enhancement involves a different part of the immune phenomenon from that which gives rise to the marked suppression of antibody because it only occurs when irradiation is administered *after* the injection of antigen, i.e., after the immune process has been initiated and after it has passed the point of radiosensitivity which leads to the marked or complete suppression of antibody formation.

Enhanced antibody formation could conceivably be the resultant of a stimulation superimposed upon the injury which is associated with a slowing of the general immune process. In view of the fact that it occurs shortly, if not immediately, after the period most sensitive to X rays (fig. 1), it would seem more likely that it represents the removal of some limiting factor and, thus, permits an injured antibody-forming mechanism to continue until it produces above normal amounts of antibody. It suggests (see Taliaferro et al, 1952) the metabolic stimulation of cells often observed with low concentrations of cellular poisons or during the early action of high concentrations.

The radiosensitivity of the anamnestic response is clearly evident and is in complete agreement with our earlier studies with Janssen (1952).

CONCLUSIONS

Using rabbit antisheep hemolysins photometrically determined in 50%

hemolytic units, a further study has been made of the radiosensitivity of the antibody-forming process in rabbits immunized with sheep red cells and administered 500 r total body X irradiation.

1. When 0.125 ml of 1% red cells were injected after irradiation, the ability of rabbits to form hemolysins rapidly fell. Thus, mean log peak hemolysin titer was not significantly suppressed when antigen was injected ½ and 1 hour postirradiation, was significantly decreased at 4 hours and continued to decrease for the following 20 hours. Recovery was rapid for the first week and was complete by the 8th week. A probit plot of percent suppression (attainment of log peak titer of 2.2 or less) against log of postirradiation time indicated a median recovery time of 4.05 ± 1.6 days.

2. A general retardation of the immune response occurred without a significant decrease in the mean log peak titer when antigen was injected from 4 days preirradiation to 1 hour postirradiation. When irradiation was administered at the end of the induction period, i.e., 4 days after the injection of antigen, the rate of antibody rise to peak titer was decreased and the length of rise increased.

3. The enhancement of log peak titer, which was superimposed on the general retardation noted in 2, occurred when antigen was injected before irradiation. It involves a later stage of the immune process than the one giving marked suppression and is probably similarly limited to a short interval.

4. The anamnestic response using an antigen dose of 1 ml of 10% sheep cells /kg rabbit was as radiosensitive as the primary response.

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STUDIES OF THE LOSS OF VIABILITY OF STORED BACTERIAL AEROSOLS

I. *MICROCOCCUS CANDIDUS*

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That infections may be airborne has been abundantly shown and this has already been discussed by us (1949 and 1951). That they should be related to bacterial or viral survival during or after drying seems clear. That bacterial survival, in turn, depends not only upon species but also upon environmental conditions has also been demonstrated. Among the latter are temperature, humidity and the state and composition of the medium from which drying occurs. The individual effects of these independent variables have, however, received scant experimental consideration, and the composition of the medium has been poorly defined chemically. In earlier studies of bacterial aerosols direct experimental differentiation between decrease in the number of viable particles due to settling and to actual loss of viability was not convenient. Consequently, interpretation of results has been difficult.

Using the method already described by us (1951), it is possible to measure (1) the concentration of viable organisms and (2) the total concentration of particles of bacterial size. The ratio of these quantities yields an estimate of viability; the latter can be used to estimate the effect of settling. In this way we have initially studied loss of viability of *Micrococcus candidus* in aerosols generated from suspensions in simple chemically defined mediums after storage under controlled atmospheric conditions. The organism is a small, spherical saprophyte, growing chiefly as singlets and doublets and readily enumerated by the Gucker photoelectronic counter (1947). It is both hardy and harmless.

The method as described has two limitations. (1) Total particulate concentration should, because of physical characteristic of the photoelectronic counter, not exceed 2000 per L. (2) The concentration of solids in the suspending medium should not materially exceed 0.15 g per L. Droplets from more concentrated mediums will, upon evaporation, produce residual particles that can be counted, thereby invalidating this method of measuring total bacterial concentration. Although these limitations have been respected in the work reported, they can presumably be overcome and thus give the method wider application.

Although our results are frequently variable, they suggest that without careful control and study of single independent variables, progress is likely to be uncertain.

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METHODS

General.—Ideally, readily interpretable studies of the viability of bacterial aerosols entail observance of the following conditions: (1) Each particle should contain only one organism. This necessitates homogeneous dispersal. (2) Decrease in the number of viable particles due to settling should be separable from loss of viability. (3) The physical-chemical environment surrounding the

particle, including temperature, pH, relative humidity, and composition of the medium surrounding the organisms, should be known. Initially the composition of the medium should be simple, since interactions will presumably be more likely in more complex mediums. (4) Sampling and measurements should be so rapid that decay of viability can be accurately followed. (5) Hazards due to leakage resulting in possible infections should be minimized. This precaution is rigorously necessary for the contemplated extension of similar studies to pathogenic bacterial aerosols.

The specific procedures described below appear to satisfy most of these criteria completely. Although some particles contain more than one organism, their proportion is generally not excessive. It will, however, be shown that other methods will be necessary for the effective study of rapid decay processes.

Bacterial aerosols at concentrations generally ranging from 1000 to 2000 particles per L aerosol have been generated by atomizing aqueous bacterial suspensions containing 10^8 to 10^9 organisms per ml using slight modifications of methods already described (1951). Although the bacteria were generally suspended either in distilled water or dilute salt solutions, including buffers, a few experiments are reported in which serum albumin or riboflavin were added to the medium, as well as one series in which broth was the suspending fluid.

a. Bacterial suspensions were prepared from 18-hour cultures at 25 C in tryptose broth containing 0.3% glucose. Growth in 4 centrifuge bottles each containing 175 ml resulted from an inoculum containing about 1/10 of the growth on an agar slant. For the sake of uniformity the turbidity of the inoculum was standardized with a Coleman Universal Spectrophotometer. After three centrifugations and two washings with distilled water, the combined precipitates were resuspended in 30 ml distilled water, and then stored in the cold room until the day of use. Although viability did not appreciably decrease during 12 days,* suspensions were usually used within a week of their preparation. There appeared to be no correlation between age of suspension and initial viability or rate of viability loss in the aerosols produced.

Finally, 4 ml of concentrated suspension were brought to 200 ml with an appropriate diluent and mechanically mixed for 10 minutes by means of a homomixer,† which produced no detectable loss of viability.

The diluents were distilled water, or solutions at ionic strength 0.0017 containing either NaCl, KCl or buffers at the ionic strengths indicated.‡ The buffers selected seemed unlikely to be toxic and were as follows:

PH range	*Buffer system*	*Ionic strength*
7.2–8.6	Glycine-NaOH	0.00034
6.2–7.6	K_2HPO_4–KH_2PO_4	0.0017
4.2	β-alanine-HCl	0.00034
3.6–5.8	Acetic acid-sodium acetate	0.0017

The pH of diluted suspensions was determined electrometrically.

b. "Apparent viability of suspensions in per cent" used for comparison with the viability of unstored aerosols was calculated from the ratio

$$\frac{\text{number of viable colonies per ml}}{\text{total number of organisms per ml}} \times 100,$$

after estimating the numerator from colony counts of plate cultures from diluted samples, the denominator by direct counts in a Petroff-Hauser chamber. This ratio generally increased during the first 25 minutes of atomization, presumably due to the breakup of particles containing more than one bacterium. Subsequently, no significant decrease was observed within 55 minutes. Generation of aerosols for storage was therefore begun about 25 minutes after the atomizer was turned on; it ended about 20 minutes later.§

c. Mean number of organisms.—This was determined from differential counts in which the number of particles containing 1, 2, 3, 4, and more organisms was counted. Generally 55 to 70% of the organisms appeared as singlets, and the mean number per particle in the experiments subsequently reported did not exceed 1.3, unless so specified. The mean number did, moreover, decrease during the course of reflux atomization.

AEROSOL GENERATION AND STORAGE

a. Generation.—Aerosols were generated by methods already described (Ferry, 1949 and 1951). Compressed air at about 35 p.s.i. was obtained from a service line, dried over silica gel,‖ and passed through two Mason-Neilan reducing valves in series, to enter the generating and

* With other organisms viability decreased demonstrably during this period.

† Laboratory Homomixer #1 Eppenbach, Inc., Long Island City, New York.

‡ For details consult Cohn (1927, 1938, 1943) and Green (1928).

§ The procedures for the preparation and evaluation of suspensions are also applicable to *S. marcescens* and *Escherichia coli*. With *Myobacterium phlei* homomixing increases clumps.

‖ Lectrodryer Dehumidifier type AAC, size 25, Pittsburgh Lectrodryer & Co., Pittsburgh, Pennsylvania.

storage system at about 15 p.s.i. This is indicated at the upper left of figure 1. Copper supply lines led to needle valves (V_1 to V_4 inclusive) all followed by filters. One of these, V_1, and a manometer served to control and meter the flow of atomizer air. This passed successively through a filter, tempering coil, humidifier bottle, and trap to the atomizer. At 5 p.s.i. flow varied from 5 to 9 L per minute, depending on the atomizer used. In these experiments the atomizer was of the reflux type.* Connection with a motor-driven syringe served to supply enough water (about 0.05 ml/min) to compensate for evaporative loss within the atomizer.

As saturated mist emerged from the atomizer it was immediately diluted with filtered and tempered mixing air flowing at 32 L per min. Flow was manually adjusted by means of a calibrated capillary flow meter and V_2.

After passage through a mixing tube, 0.8 to 1.5 L of the concentrated aerosol or "smoke" was aspirated by Venturi action and diluted with 41 L of conditioned air which determined humidity of the aerosol. Excess "smoke" could, if necessary, be readily sterilized before it was vented into a stack. Part of the diluting air was nearly saturated by passage through a thermoregulated humidifying column about 48 inches long. Humidity was adjusted by controlling the ratio of nearly saturated to dry tempered air by means of a flow meter and the needle valves V_4 and V_3, respectively. In practice, V_4 was first opened to permit the necessary flow of saturated air; subsequently, the volume flow was brought to 41 L with V_3.

After passage through a mixing tube, the conditioned aerosol was ready for sampling by photoelectronic or biological methods, or for storage. Since all parts of the system, except control valves, filter, and the humidified column, were immersed in water baths thermostatically controlled to ± 0.05 C, and since, with practice, flows could be kept constant, relative humidity of the unstored aerosol could be regulated within 2 to 3% at 15 and 25 C. At 35 C regulation is less certain. We estimate that the unstored aerosol is not over 2 seconds old.

In passing through the generating system physical changes in the aerosol can be envisaged. Since larger droplets were trapped by impingement, we assumed a droplet diameter of 5 to 10μ in the emergent mist.† Of those droplets containing particles, 95% presumably contained only a single particle when concentration in the suspension, largely singlets, was about 10^8 per ml. During dilution evaporation was rapid, so that the emergent aerosol contained only nearly naked organisms and droplet residues too small to be counted. From observed atomizer delivery rates, concentration of droplets in the mist and of all particles in the aerosol should not greatly exceed 10^4 and 50 per ml, respectively. Under these conditions, aggregation seems unlikely, so that the mean number of organisms per bacterial particle in the aerosol should be no greater than in the aqueous suspension. These assumptions are qualitatively confirmed by observations of traces of impulses in a cathode ray oscillograph, which suggest that smaller particles, presumably singlets, predominate.

b. Storage system.—As shown on the right of figure 1, this consists essentially of a cylindrical, water-jacketed shell in which a rubber balloon,‡ serving as a collapsible storage chamber, is suspended. The capacity of the shell is about 700 L, that of the balloon 400 L. The former was fabricated from type 304 stainless steel. Tests had demonstrated that it was free from leaks and could withstand 60 p.s.i. internal pressure.

Suitable connections provide for (1) inserting the balloon; (2) introducing aerosol; (3) the supply or removal of "control" air; (4) circulating thermoregulated water through the shell, and finally, if need be, (5) steam sterilization. The latter two are not shown. Appropriate manipulation of valves 5 to 7 inclusive permits either the entrance of conditioned air from a supply line or its removal by a vacuum pump. Noteworthy are first, an overflow with V_6 marked "excess" in the diagram, which serves to keep the pressure between the balloon and shell only slightly above that of the atmosphere during sampling; and second, a similarly marked side tube on mixing tube 2, which serves the same purpose in the aerosol storage and sampling system; both tubes can be led to an exhaust stack.

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* This type of atomizer is unsuitable for use with suspensions of organisms which are fragile or clump readily. With them one can use a similarly designed, nonreflux atomizer supplying the liquid jet directly at about 2 to 10 ml per minute. Provision must also be made to drain off fluid which results from the accumulation of larger droplets within the atomizer.

† C. N. Davies. Personal communication. Using an atomizer with similar characteristics, the diameter of emergent mist droplets was about 5 μ.

‡ Darex Kytoon balloon type K125, made by Dewey and Almy Chemical Co., Cambridge, Massachusetts. Before insertion, the balloon was carefully washed and rinsed, finally with distilled water, and then dried with filtered air.

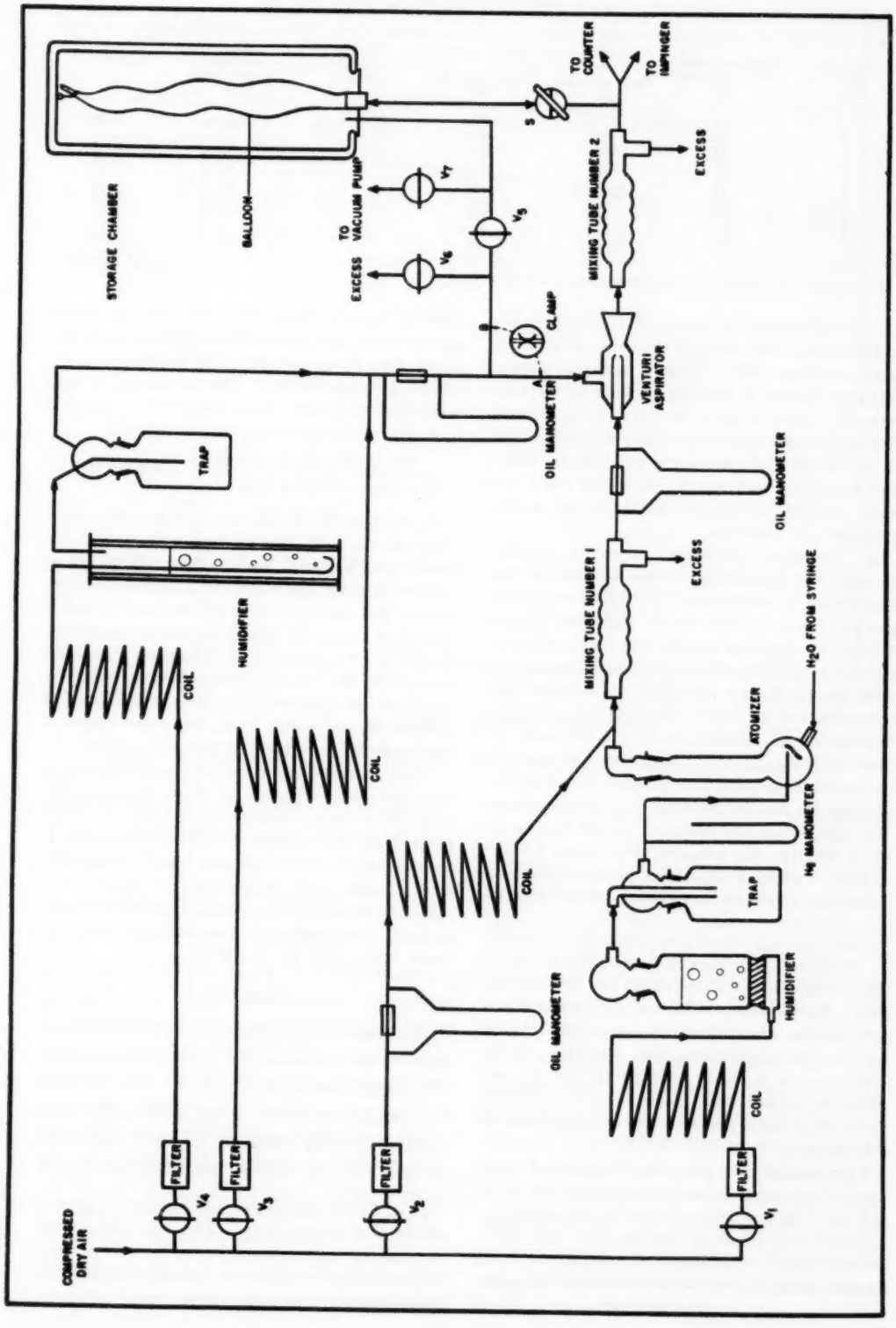

FIG. 1.—Aerosol generating and storage system.

TABLE 1.—*Protocol of experiment of 6-1-51.*

Sample no. (1)	Mean storage time, minutes (2)	Concentration per liter aerosol		Apparent viability, percent (5)	Relative viability, percent (6)	Log RV (7)
		Total particles (3)	Viable particles (4)			
1	0 Control	1170	700	59.8	100.0	2.00
2	0	1128	646	57.3	58.6	
3	13	1034	312	30.2	51.5	1.71
4	18	1049	271	25.8	44.0	1.64
5	58	930	213	22.4	39.1	1.59
6	63	947	209	22.1	37.7	1.58
7	132	814	151	18.6	31.7	1.50
8	137	845	147	17.4	29.7	1.47
9	194	689	Lost			
10	200	674	99	14.7	25.1	1.40

Temperature 25 C; relative humidity percent: initial 42.0, final 43.7.

c. Particle counter (Gucker, 1947).—Only minor modifications were made in the instrument previously described (1951). First, a cathode emission rectifier replaced a mercury rectifier in the time recorder power supply; second, an appropriate resistance-capacitance wave filter was introduced to attenuate high frequency background noise; and third, a Stancor type 752 power pack was used, instead of storage batteries, to supply vacuum type filaments.

*d. Operation.**,†—After adjusting airflow through the generating system so that air was appropriately conditioned and observing background counts which rarely exceeded 10 per minute, an impinger sample was taken to insure sterility of air. Then 10 ml of bacterial suspension were placed in the atomizer, atomization and compensation for evaporation started, and particulate counts initiated. As soon as the latter became constant, generally after about 30 minutes, the unstored aerosol was sampled for viable organisms by two jet bubbler impingers in series. The duration of the sampling period was generally 4 minutes; the measured flow about 6 L per minute, or about $\frac{3}{4}$ the critical pressure flow rate.

Another measured sample of aerosol was aspirated through three Schwartz tubes in series, each containing magnesium perchlorate to absorb water. The water content of the sample was estimated gravimetrically.‡ The accuracy of this analytical procedure was about 1%. Relative humidity (RH) was computed as follows:

$$\frac{\text{Water content per L aerosol}}{\text{Water content per L saturated air}} \times 100 = \text{RH}$$

In order to fill the balloon with aerosol a glass stopcock (S) with 10 mm bore was opened, V_7 opened and V_5 closed, and an artery clamp left in position B. At a maximum filling rate of 23 L per minute this operation took 18 minutes. V_7 and S were then closed. To deliver aerosol for sampling, V_5 and S were opened and the clamp moved to position A. The rate of delivery and pressure were regulated by adjusting V_6. After 8 samples for particulate and viable assay had been taken, a final sample was taken for humidity estimation.

Aliquots taken from the impingers, diluted if necessary, were cultured on plates in quintuplicate. From colony counts made after 24 hours incubation, the number of viable particles per L aerosol could be calculated. Total particulate concentration per L was ordinarily directly estimated from particulate count, corrected for background. The necessary computations have already been given by us (1951).

RESULTS

To illustrate subsequent treatment of results we present the data from a typical experiment in table 1. The values for mean storage time represent the sum, one-half time of filling+time between end of filling and beginning of

* During early experiments changes in humidity of aerosols were observed, presumably due to the transmission of water vapor through the balloon. These were subsequently avoided by equilibrating the storage system with "control air" at the temperature and humidity of the aerosol to be studied. Thoroughly flushing and filling the system with conditioned air, followed by letting it stand in the system overnight, served this purpose.

† Organisms and particles disappeared completely from aerosols which stood overnight in the balloon. This was demonstrated by negative plate cultures from impinger samples and zero background counts respectively. Consequently, sterilization of the system between runs was unnecessary.

‡ An Alnor dewpointer, used in earlier experiments, was unsatisfactory. This was presumably because too few condensation nuclei were present to permit the formation of a barely visible mist. This is essential with this instrument.

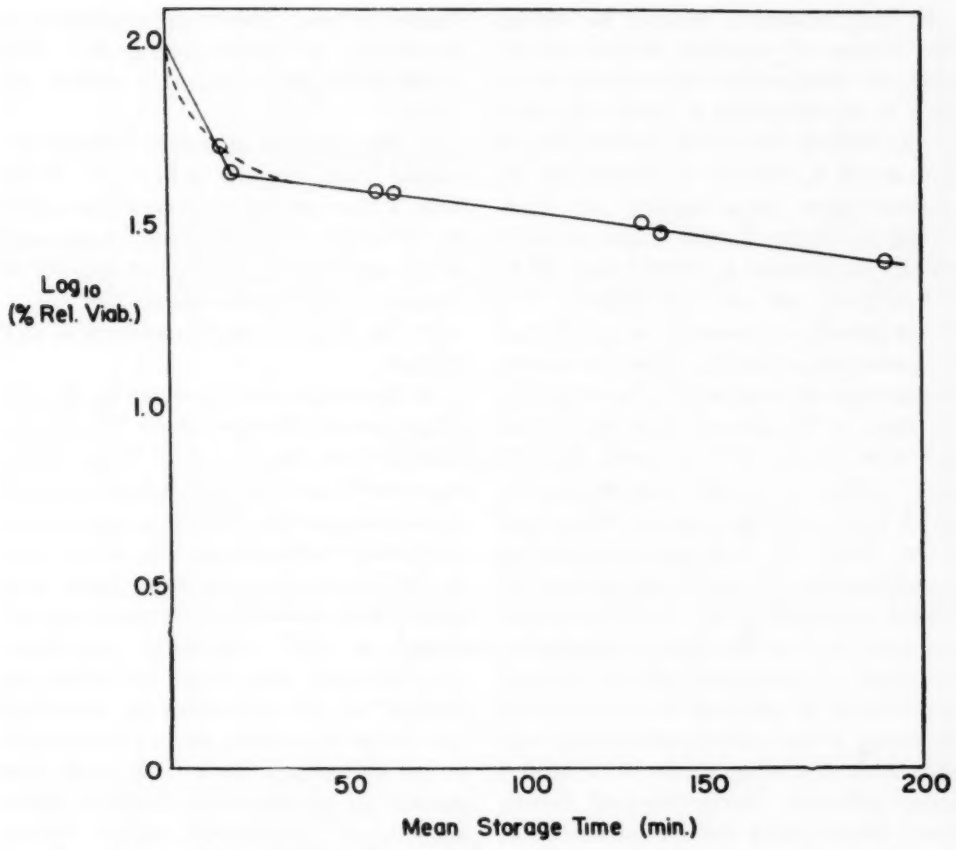

FIG. 2.—A typical experiment. Change in relative viability as a function of storage time.

sampling+one-half time of sampling. *Apparent viability* of the aerosol in percent is defined as the ratio

$$\frac{\text{number viable particles per L}}{\text{total number of particles per L}} \times 100.$$

For unstored aerosols it generally approximated the viability of bacterial suspensions used for atomization. On this account and to facilitate comparison of experiments, we have subsequently calculated results as relative viability in percent, appearing in column 6. It is defined by relative viability

$$\text{(RV)} \quad \frac{\text{apparent viability at } t_n}{\text{apparent viability at } t_0} \times 100,$$

where t_n = mean storage time and t_0 = zero storage time. For unstored aerosols RV = 100. Log_{10}RV appears in column 7.

In figure 2, values for $\log_{10}$RV appear as ordinates, those for mean storage time as abscissas. Inspection of this figure suggests that *M. candidus*, like other organisms studied by Dunklin and Puck (1948), dies off rapidly at first and then more slowly; consequently, two processes may be involved. The phenomena may, as a rough first approximation, be represented by two straight lines with characteristic slopes, each represented by the ratio $\Delta \log_{10}\text{RV}/\Delta t$. The numerical value of the ratio multiplied by 230.3 yields directly percentile loss of viability per minute. The rate for the first process we designate as k_1, that for the second as k_2.

It can, however, readily be shown that if loss of viability indeed results from two independent logarithmic processes, it should follow a curve suggested by the dotted line, with limiting slopes from which k_1 and k_2 may be derived. In representative experiments, as illustrated by figure 2, the slopes of lines which characterize k_2 extend from 20 to 180 minutes and are well defined. The initial process is, however, so rapid that it cannot be accurately observed in the storage system employed. Nevertheless, the slope of the secant from log 100 at zero time to log RV at some definite time yields a rough approximation which is always less than the true value of k_1. With *M. candidus* aerosols we have taken log RV at 17 minutes as the second reference point. This represents the mean age of the first two samples. The slope of the secant $\times 230.3$ we shall designate as k_1', and use it in comparing the rapid, initial rates of decreasing viability observed when aerosols are stored under different environmental conditions. More exact determination of k_1 necessitates a different experimental approach.*

The data from which the values for k_1' and k_2 are derived generally conform to the following criteria:

1. Particulate concentrations of unstored and of the first stored samples do not differ significantly; the balloon was, therefore, presumably empty at beginning of storage. This observation also suggests that loss by impaction is insignificant and is in accord with the theoretical treatment of Lidwell (1948).

2. Relative humidity remained constant within about 5%.

3. The apparent viability of the aqueous bacterial suspension atomized did not decrease but usually increased during a run. This was attributed to shattering of larger aggregates. This criterion is not applicable where pH <5.0.

4. The number of viable colonies obtained from aliquots taken out of the first of two impingers in series exceeded 35, even in those obtained at the end of an experiment; at earlier stages the number ranged from 100 to 250.

5. The slope of decay curves was well defined.

In figures 3a and b, values for k_1' and k_2 appear as ordinates, those for relative humidity as abscissas. Full black circles represent humidity determinations with Alnor dewpointer. All others were gravimetrically determined. The data refer to earlier experiments on aerosols generated from unbuffered suspensions and stored at 25C. Humidity regulation was, however, often imperfect. Since the change of the logarithm of humidity was linear with time, mean values could be estimated and have been used. The percentage of singlets in distilled water suspensions apparently varied rather widely about a mean of 50%. As we shall subsequently show this leads to underestimates of both constants. Three values for k_2 have been included which violate criteria 4 and 5; they are, however, compatible with the other values.

Despite the evident variability of the data we have drawn a dashed curve to represent the trend of variation in the constants with changing humidity. k_1' increases sharply when relative humidity exceeds 80%. Under these conditions evaporation should be incomplete, and leave the bacteria surrounded by a saturated or concentrated salt solution. This phenomenon, already suggested by Duklin and Puck (1948), accords with the experience of housewives, food preservers, and scientists (Ficker, 1908, Hotchkiss, 1923), who have long known that concentrated salt solutions either

* We have devised a dynamic storage system which appears to fulfill this purpose.

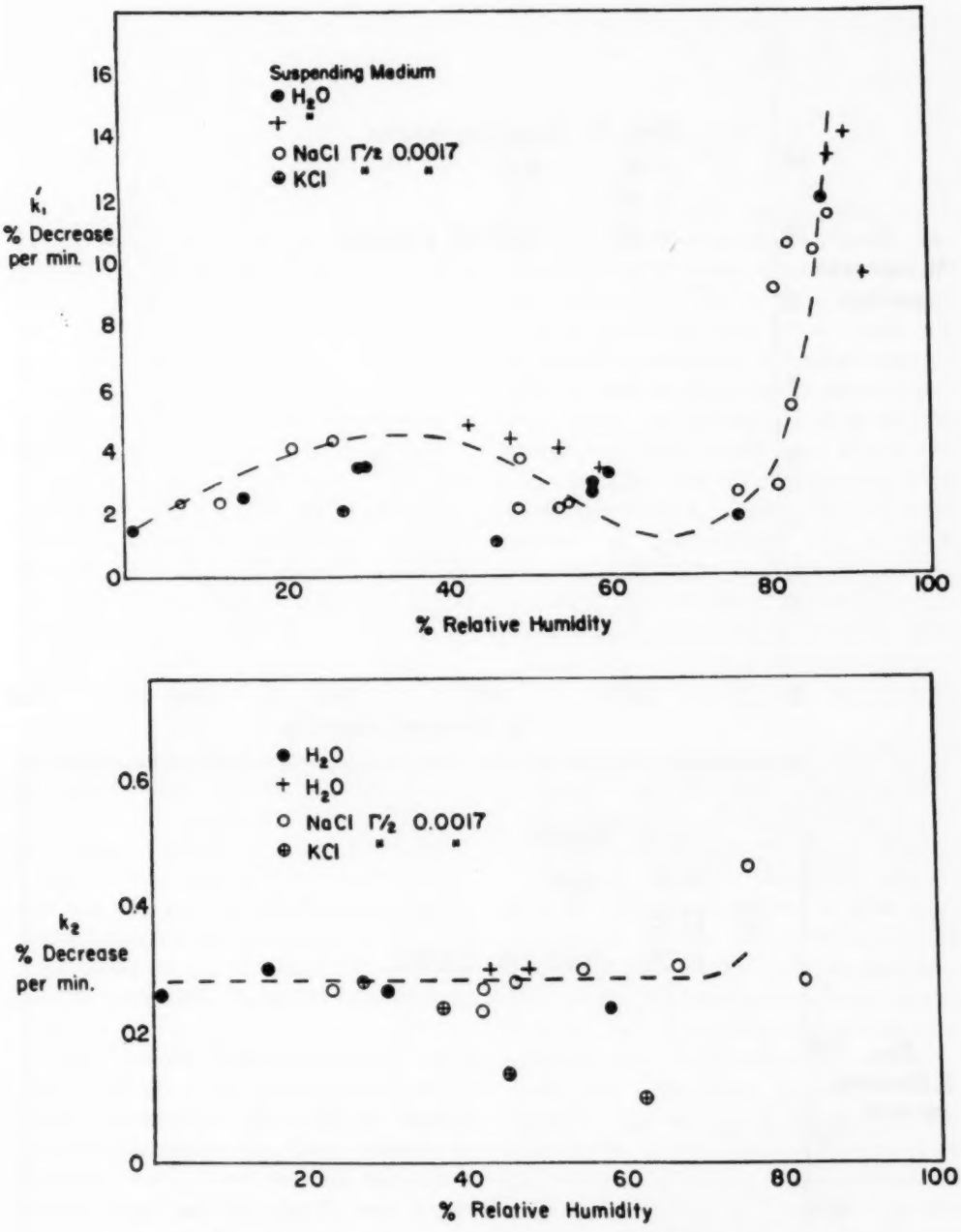

FIG. 3a and b.—Rate of viability decay as a function of humidity. a. k_1'. b. k_2.

kill or inhibit bacteria. k_2 on the contrary, seems rather insusceptible to humidity change. Although k_1' and k_2 may be slightly lower in those aerosols generated from bacterial suspensions in dilute KCl, the present data are inconclusive.

In figures 4a and b appear values for k_1' and k_2 respectively, derived from observations at 15 C and 35 C. For com-

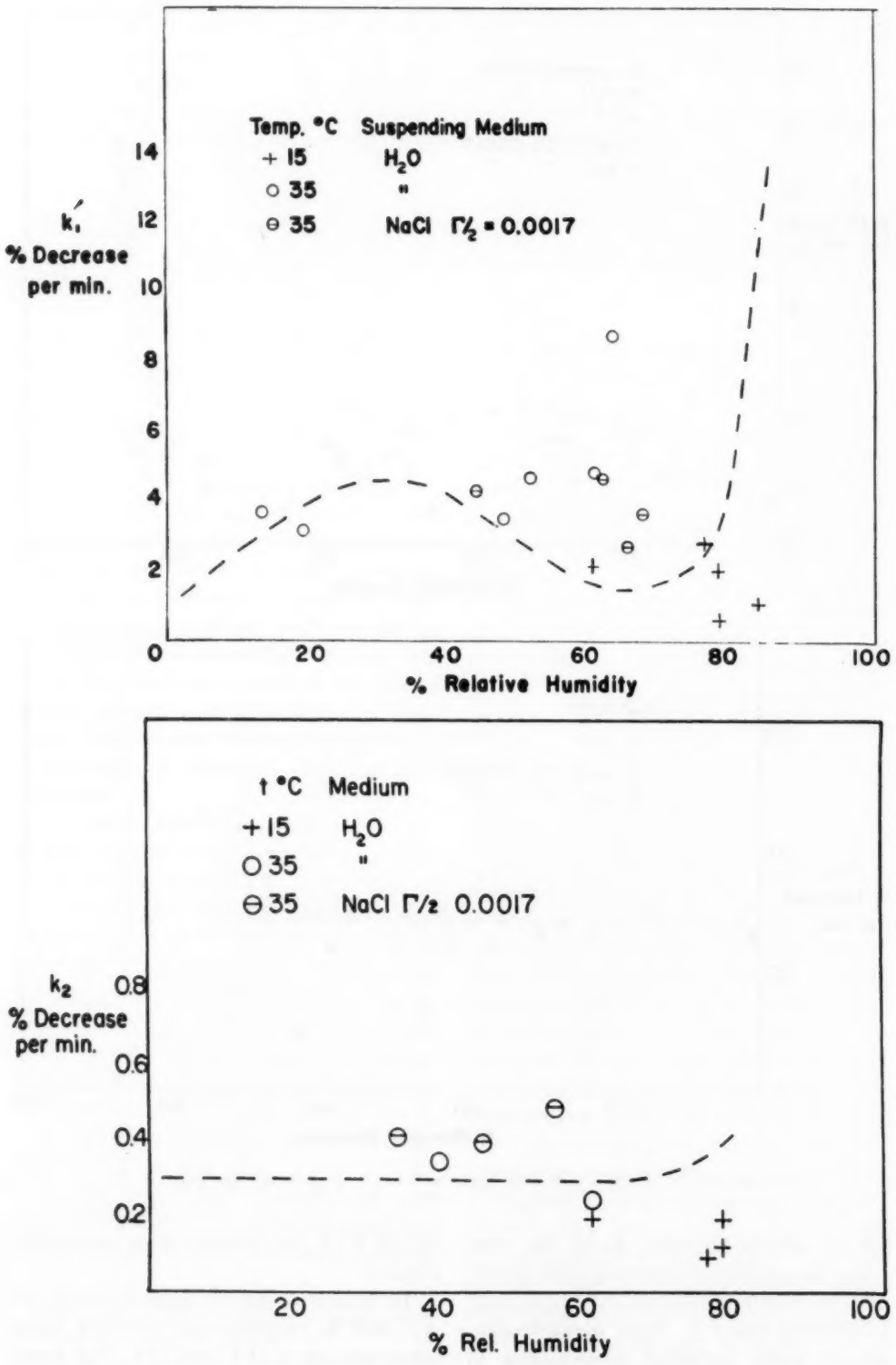

FIG. 4a and b.—Rate of viability decay as a function of temperature. a. k_1'. b. k_2.

parative purposes we have repeated the dotted curve of figure 3. Although scatter of data is great, especially at 35 C, where humidity control was less satisfactory, loss of viability is more rapid at the higher temperature. Over the temperature range 15 to 35 C, both the vapor pressure of water and the rates of chemical reactions are approximately doubled with a 10 C rise in temperature. The increase in decay rates appears to be somewhat less.

In later experiments, with aerosols generated at RH = 55 to 60% from bacterial suspensions in buffered mediums, results were more consistent. They are illustrated in figures 5a and b, in which the ordinates are k_1' and k_2, and the abscissas represent pH of the suspending medium as indicated by the symbols. Buffers were generally at ionic strength 0.0017, added riboflavin at either 0.01 or 0.1 g per L, and human serum albumin at 0.1 g per L. In the case of McIlwain's buffer the total concentration of citrate and phosphate was about 0.001 *M*. The points generally represent averages of three or more determinations; in the case of phosphate buffer, pH 6.5, and glycine, pH 8.6, two results were averaged. With glycine at pH 7.2 and broth at pH 5.0 and pH 4.6, the points were each obtained from a single run.

The "errors of measurement" have been computed for points which represent the average of replicate experiments, excluding the data relating to acetate buffers for reasons explained below; they are ± 0.7 for k_1' and ± 0.08 for k_2.

The data concerning aerosols generated from bacterial suspensions in broth deserve special comment. Since broth droplets produced by atomization evaporated and gave rise to a particulate concentration which could not be photoelectronically determined, it was possible to measure only the number of viable particles. The logarithm of this number, plotted against time, appeared to approximate two straight lines with definite slopes, each representing the sum of a settling rate, k_s, and k_1' or k_2, respectively. In the case of other aerosols studied the rate of decrease of particulate concentration was also logarithmic with time. But even if settling of residual particles from broth could not be directly estimated, it seemed reasonable to assume, as a rough approximation, that glycine-coated and broth-coated material would have about the same density and size distribution and about the same k_s. Accordingly we subtracted k_s, determined for aerosols generated from suspensions in glycine, from the rates of decrease in viable particulate concentration observed in aerosols generated from broth suspensions. These appear in figure 5 as k_1' and k_2.*

All points except those relating to aerosols generated from acetate suspensions at pH 3.8 and k_2 for broth at pH 5.0 approximate the curves as drawn.

Since the acetate buffered suspensions at pH 3.8 contained a large proportion of volatile free acetic acid, pH may well shift to the right during drying. At pH 3.8, moreover, clumping occurs so that observed k values are presumably low. The errors of measurement are, therefore, greater. Consequently, the points should be shifted diagonally upward, as indicated. The points for k_2 relating to suspensions in phosphate buffer at pH 7.0, and the broth at pH 5.0 are off the curve. Except

* Although k_1 and k_2 are thus indirectly estimated, comparatively slight modifications in experimental technic should make the method more generally applicable. It should thus be possible effectively to study aerosols with higher particulate concentrations. This hypothesis deserves further verification.

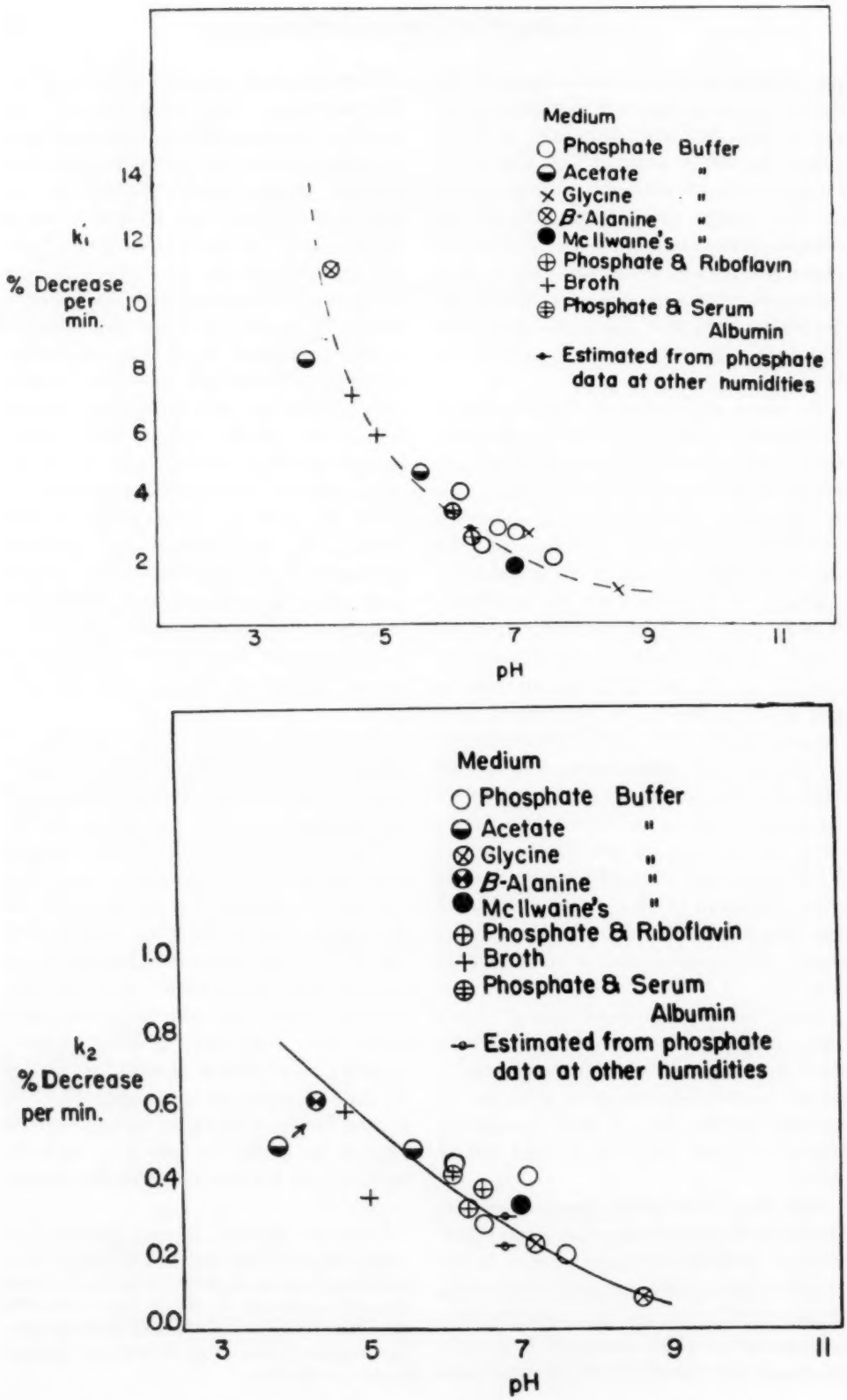

FIG. 5a and b.—Rate of viability decay at about 60% RH as a function of pH. a. k_1'. b. k_2.

TABLE 2.—*Percentile loss of viability per minute of atypical* M. candidus *aerosols generated from suspensions in phosphate buffer.*

Suspension					Aerosol					
					Decrease in viability per minute, percent					
					k_1'			k_2		
						Corrected			Corrected	
Date	pH	Age in days	Singlets, percent	Relative humidity, percent	Apparent	For clumping	To 60% RH	Apparent	For clumping	To 60% RH
2-19-51	6.8	4	57	50	2.7			0.60		
2-21-51	6.8	6	69	49	3.8			0.41		
8-15-51	6.7	6	39	50	4.6	5.7		0.25	0.32	
					Avg. 3.7	4.1	2.7	0.42	0.44	0.30
8-17-51	6.8	1	37	41	4.0	5.3	2.7	0.30	0.43	0.22
8-9-51	6.6	7	51	56	3.9			0.33		
8-13-51	6.6	4	34	58				0.28	0.37	
8-21-51	6.7	3	31	56	3.3	4.6		0.25	0.32	
9-17-51	6.8	4	34	56	1.7	2.9		0.21	0.39	
9-19-51	6.9	6	26	54	3.0	3.8				
Avg.	6.7				Avg. 3.0	3.7		0.27	0.35	

for the possibility of experimental error, there is no ready explanation for this.

The bacterial suspensions used in the experiments represented in figures 6a and b were at pH 6.4; the humidity varied. The crosses are taken from the curves of figure 5; dots are directly derived from experiments. Both k_1' and k_2 appear to pass through a minimum at about 75% with no maximum. It is, however, rather surprising that k_2 does not increase as markedly as k_1' at higher humidities. Since humidity was somewhat less during the latter part of the experiment, the true increase may be greater.

In table 2 appear data for a series of runs, which were at first difficult to interpret, since they seemed not to accord with results from earlier experiments, which they were intended to confirm. In fact, they differ from these experiments either because the dispersion of bacterial suspensions was poor, or the humidities at which aerosols were stored were lower, or in both of these respects.

Whenever the bacterial suspension contained a small proportion of singlets we believed that at least some of the smaller groups of organisms appeared in the resulting aerosol. Moreover, the probability that a group contained a viable organism, thereby giving rise to a colony on a culture plate, increased with the number of organisms in the group. It followed that particulate viability would exceed the viability of single organisms. The distribution of singlets, doublets, and triplets in the suspension was, however, known, and the mean number per particle calculable. The actual distribution appeared, within the experimental error, to satisfy the requirements of a modified Poisson or geometric distribution equally well.

Provided the mean number of organisms per particle and apparent particulate viability are known, one can compute the mean viability of single organisms applying simple statistical considerations based on either type of distribution. The results are graphically shown in figure 7.*

Applying the reasonable assumption that the distribution of bacteria in aerosol particles accurately reflects distribution in the suspension from which they were generated, we can, by interpolation between the curves, derive corrected estimates of the viability of single organisms from particulate viabilities of samples.

From such estimates we have derived

* This treatment was kindly developed for us by Professor Hugo Muench, Professor of Biostatistics at the Harvard School of Public Health

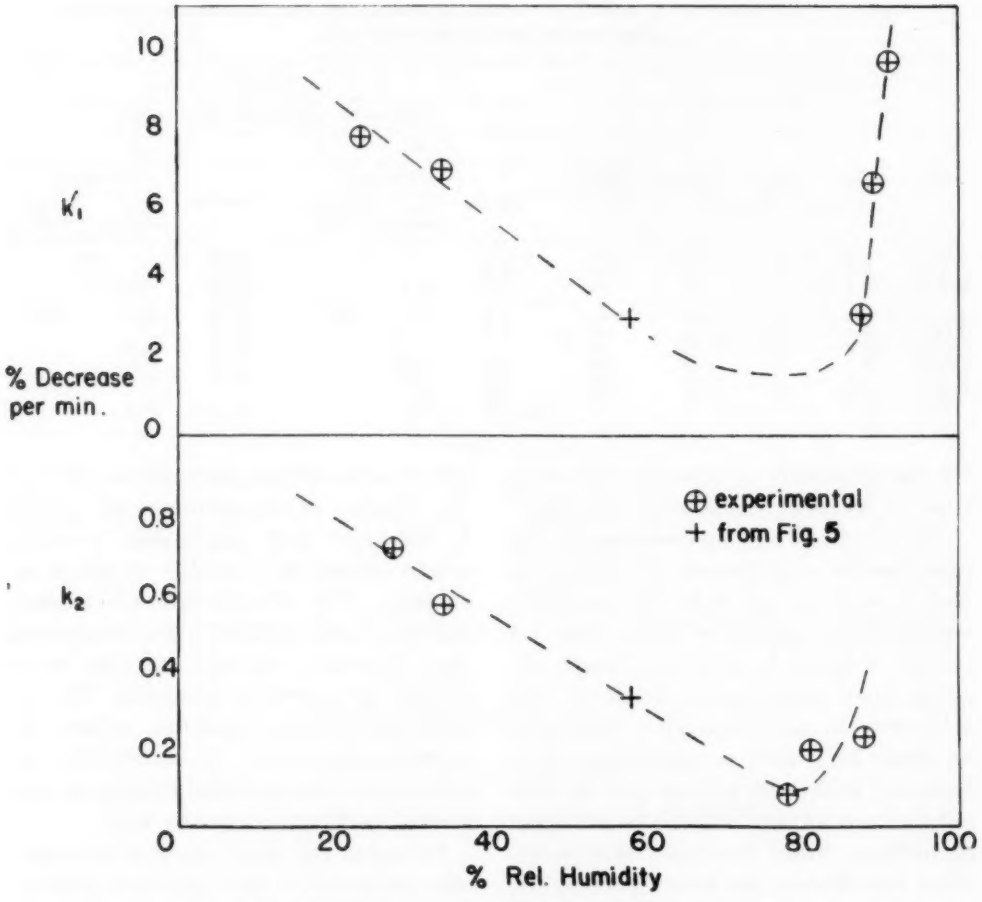

FIG. 6a and b.—Rate of viability decay at pH 6.4 as a function of humidity. a. k_1'. b. k_2.

corrected values for k_1' and k_2 generated from suspensions containing material less than 50% singlets. These appear in table 2.

The corrected constants pertaining to aerosols stored at about 56% RH would lie slightly above the curves of figure 5. Aerosols stored at 50 and 41% RH, respectively, yield constants that are, as might be expected, rather high. Assuming that the constants obtained at pH 6.8 were affected by humidity in the same manner as those obtained at pH 6.4, we corrected these values, using the curves of figure 6. The values, corrected to 60% RH, appear in table 2 and are shown in figure 5 under the caption "estimated from phosphate data at other humidities." In view of the approximations and assumptions involved, agreement with the curve is satisfactory.

DISCUSSION

Earlier studies of bacterial aerosols by Williamson and Gotaas (1942) and DeOme et al (1944), and of aerosols containing influenza virus by Edmonds et al (1943) and Loosli et al (1943) showed that the death rate per minute rose with increasing humidity. Wells and Zappasodi (1942), however, reported a contrary finding with hemolytic streptococcus.

Subsequently Dunklin and Puck

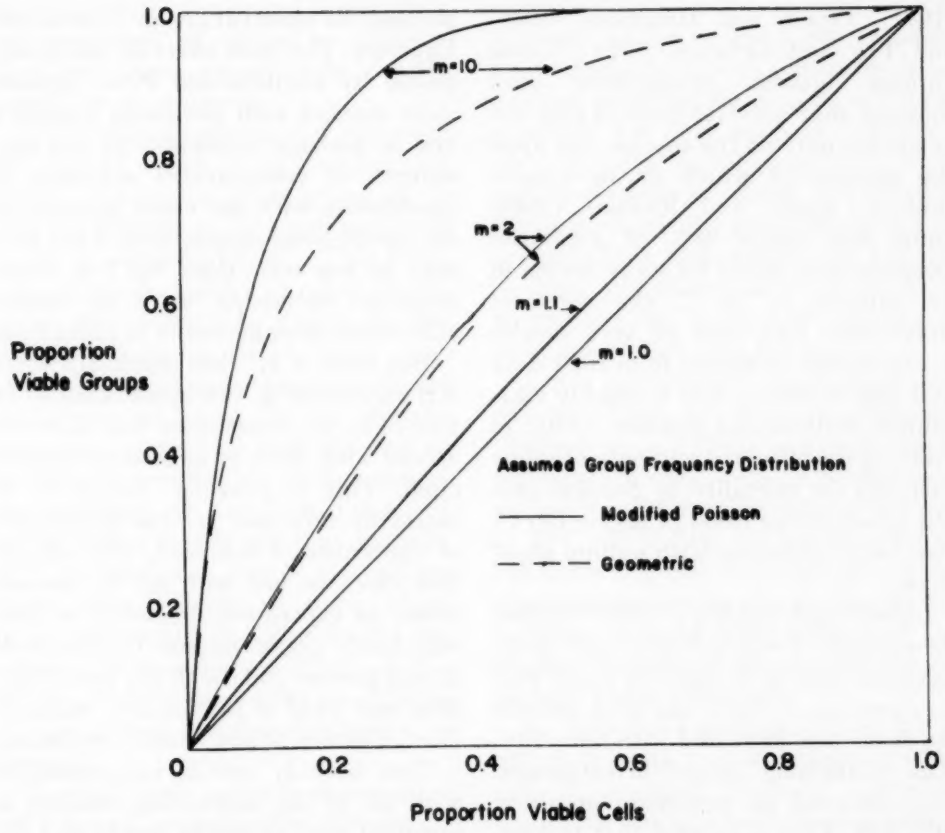

FIG. 7.—The estimation of viability of single cells from the observed viability of groups containing a mean of 1 to 10 cells. m = mean number of cells in group.

(1948) found that loss of viability of pneumococcus, *Streptococcus hemolyticus* type C and staphylococcus aerosols occured in two stages, the first rapid, and the latter slow. Plotting logarithm of viability against time, the data were represented by two straight lines with slopes characterizing two death rates. They also observed that except for aerosols generated from bacterial suspensions in distilled water, the rates were maximal between 50 and 60% RH. This effect they associated with a toxic effect of NaCl. By studying the disappearance of methylene blue from aerosols, they attempted to distinguish between apparent and real decreases in viability due, respectively, to particulate settling and death, and death alone. In some cases the secondary death rate, at either low or high humidities, was so small that estimates, corrected for settling, appeared as negative values.

Subsequently Schechmeister (1950) obtained a minimal recovery of influenza virus from aerosols at 60% RH. Later Schechmeister and Goldberg (1951) suggested that the apparent viability of aerosols is related to the degree and homogeneity of dispersion, as well as to the composition of the suspending medium.

Data from studies concerning the death rate of dried bacterial suspensions appear germane and qualitatively comparable with the secondary death rate of aerosols. Earlier literature has been reviewed successively by Stamp

(1947), Proom and Hemmons (1949) and Fry and Greaves (1951). These authors present quantitative data showing that survival after drying depends not only on the species, but upon the medium in which drying occurs. Bullock, Keefe, and Rawlins (1949) found that about 98% of *Aerobacter aerogenes* were killed by spray drying in air, initially at 70 C; the survivors stored over P_2O_5 died off very slowly. It can readily be shown from their data that this secondary rate is roughly logarithmic. Bullock and Rawlins (1950), in assaying the effects of a variety of saline mediums on mortality of *Bacillus subtilis* spores spray dried at about 190 C, observed a minimum with sodium phosphate.

Lidwell and Lowbury (1950) studied the survival of staphylococci and streptococci in dust. Although they found that over periods of weeks and even months the death rate increased with humidity, thus confirming other investigators, they observed no maximum at about 50% RH. They suggested that this departure from the results of Dunklin and Puck might be due to the dryness of the dust studied.

Our earlier data from aerosols generated either from suspensions in distilled water or in NaCl or KCl solutions can generally be only qualitatively compared with those of workers already cited. Differences in degree and homogeneity of dispersion, in concentration, composition and pH of the suspending medium, and variability of our results, as well as those of others, generally preclude quantitative comparison. Like those of Dunklin and Puck, our results suggest two processes affecting the death rate of bacteria in aerosols. Since they do not suggest a maximum value for k_1' or k_2 for aerosols generated from suspensions, either unbuffered or buffered at pH 6.4, they in this respect parallel the observations of Lidwell and Lowbury. The toxic effect of NaCl, suggested by Dunklin and Puck, appears more marked with increasing humidity and is, perhaps, explicable by the persistence of concentrated solutions in equilibrium with the vapor pressure of the surrounding atmosphere. That KCl may be less toxic than NaCl is, moreover, not surprising, since the former salt occurs more generally in plant cells.

But even if k_1' rises markedly when RH exceeds 80%, this appears not to be true of k_2, an observation that does not accord with those of most investigators cited. This is puzzling. For if k_1' is markedly increased by the persistence of concentrated solutions, why should this effect be less with k_2? To assume either an experimental artefact or that only hardy organisms survive the rapid drying process and die slowly thereafter, does not yield a satisfactory explanation. Further study seems necessary.

That both k_1' and k_2 vary regularly with pH of the suspending medium is apparent from figures 5a and b; that the parameters of these curves are different is suggested by the fact that the ratio k_1'/k_2 is not constant over the entire pH range covered by our studies. Its value is, approximately, 10 over the more limited range, roughly pH 6.0 to 7.0. Since k_1' is smaller than the real constant k_1, the ratio k_1/k_2 must be even greater. This is not surprising, since chemical change occurs much more rapidly in solution than in the dry state.

Consideration of the nature of the drying process also suggests that k_1 may change during drying. In our experiments rapid changes in concentration, usually from an ionic strength 0.0017 to the concentrations of various saturated solutions, occur, and with them shifts of at least 1 pH unit are possible. These changes make quantitative interpretation difficult. The resistance of *M. can-*

didus to rapid change in osmotic pressure may well be related to spherical shape and what appears to be a rather homogeneous structure.

It is rather surprising that even over the limited range of suspending mediums used by us, composition of the medium appears to be an unimportant factor in determining k_1'. This divergence from the results of other investigators may perhaps be due to the predominant importance of large, rapid osmotic changes under the experimental conditions employed by us. Further experiments in which both the concentration and composition of the suspending mediums are varied might be expected to clarify this apparent anomaly; that the mediums should be buffered seems clear. The use of more concentrated suspending mediums should, presumably, reduce changes in pH and also in concentration during drying. It is not surprising that k_2 is less affected by the medium.

The data from aerosols generated at pH 6.4 again emphasize the difference in magnitude between k_1' and k_2; the ratio k_1'/k_2, except at humidities greater than 85%, appears to be 10. This disparity in rates suggests that these rate constants may characterize two different processes. The first we tentatively relate to change in concentration with consequent change in osmotic and chemical activity; the latter may be due to slow oxidation. This hypothesis can be experimentally tested, as will be shown in a subsequent paper.

Although one could empirically derive equations for k_1' and k_2 at constant RH with pH as the independent variable, or at constant pH, with RH as the independent variable, the data do not seem adequate for this purpose. Provided adequate data, including those for thermal effects were available, it is possible that a nomographic treatment could be devised. This task would, we believe, use time and effort that could be more profitably given to the study of aerosols containing other organisms.

A few quantitative comparisons are possible. Data for dried *A. aerogenes* (Bullock et al, 1949) stored at room temperature over P_2O_5 yield the estimate $k_2 = 0.005$; for *M. candidus* aerosols at 25 C, 20% RH, pH 6.4, k_2 is 0.8 or 160 times as great. Although part of the discrepancy may be due to species differences, extreme desiccation and the method of drying in the former case are perhaps more important.

In table 3 we compare values for k_1 and k_2 relating to *Staphylococcus albus* and *M. candidus*. We have multiplied Dunklin's values, which are logarithmic, by the factor 230.3 to provide percentile rates directly comparable with ours. Since these organisms are morphologically and culturally similar, and both are hardy, species differences are probably secondary. And making the likely assumption that pH of *Staph. albus* was between 4.6 and 6.4, it is not surprising that the k_2 for each of these organisms is of the same order of magnitude. The trends are apparently different.

In conclusion, we should first stress the variability of our data, despite care-

TABLE 3.—*A Comparison of decay constants.*

Organism:	*Staph. albus*		*M. candidus*			
Suspending medium:	Broth		PO_4 buffer pH 6.4		Broth pH 4.6	
Observer:	Dunklin		Ferry			
RH%	k_1	k_2	k_1'	k_2	k_1'	k_2
30	5.3	0.69	7.0	0.65		
50	8.0	3.4	3.8	0.38		
60	18.5	0.69	2.3	0.27	4.6	0.44
80	7.3	0.69	1.5	0.10		

ful precautions to maintain controlled and reproducible conditions. Although it takes only 1 to 2 seconds for the aerosol to pass through the dilution system, it is possible that lack of humidity control in the first, or mixing stage, may be partly responsible for scatter. Should this be true, control of this variable may improve consistency of data. Nonetheless, some of the variability must still be ascribed to as yet unknown differences in the suspensions used for aerosol generation.

Second, even with hardy organisms like *M. candidus* the first decay process is too rapid to be followed with the static storage system used. For accurate measurements of a process occurring in fractions of seconds or seconds instead of minutes, a different type of system, to be described later, will be necessary. Nevertheless, estimates of k_1' made with the static system appear comparable.

And finally, further studies of aerosols including those of other organisms, as well as of aerosols generated from a variety of more concentrated mediums, appear necessary for a better understanding of one of the factors underlying infection through the air.

SUMMARY

1. Methods are described for studying the viability of bacterial aerosols during storage at known temperatures and relative humidities, following generation from suspensions in simple, chemically defined mediums.

2. The loss of viability of *Micrococcus candidus* aerosols is consistent with the assumption of two logarithmic decay processes with rate constants k_1 and k_2, respectively.

3. The first process is too rapid to permit more than approximate estimates of k_1 with a static storage system; provisional estimates are indicated by k_1'.

4. The apparent ratio k_1'/k_2 is about 10; the exact value is certainly higher.

5. Systematic variation of k_1' and k_2 has been observed with pH, at pH 6.4, where k_1' and k_2 decrease from 20 to 75% RH and pass through a minimum at about 80% RH, k_1' rises abruptly at higher humidities, k_2 only slightly.

6. The rise of k_1' when RH > 80% may be related to the persistence of concentrated salt solutions in equilibrium with water vapor.

7. It is suggested that the first rapid process may be related to rapid changes in concentration during drying, the latter at least in part to slower oxidative processes. This hypothesis can be tested.

8. Further studies of aerosols containing other organisms, of aerosols generated from bacterial suspensions in more concentrated mediums, and of the rapid initial process by means of a more appropriate method appear necessary.

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THE EFFECT OF CORTISONE AND SOMATOTROPHIC HORMONE ON VACCINIA VIRUS LESIONS AND BENZYL ALCOHOL TOXICITY IN THE EMBRYONATED EGG

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The effect of cortisone on the course of experimental infections is, in general, to enhance their severity (Freeman et al, 1950; Michael, 1950; Abernathy, 1951; editorial, 1951a and b; Glaser et al, 1951; Kilbourne, 1951a and b; Kligman et al, 1951; Redaelli et al, 1951; Thomas, Mogabgab, and Good, 1951; Turner 1951; Syverton et al, 1952).

Scherr (1953a) proposed the hypothesis that cortisone may elicit either a deleterious or efficacious effect on certain infectious diseases, depending upon the conditions and environment of the host. Thus, he reported (1953b, c, d, e) that cortisone acetate would enhance the severity of a mild infection of moniliasis in mice, would not alter the course of a moderate infection, and would reduce the severity of a severe infection. Duffy and Morgan (1951) found that rabbits injected with *Shiga dysenteriae* endotoxin showed a reduced or an aggravated febrile response to cortisone, depending upon the time-dose relationship between the toxin and the cortisone. Similarly, Kilbourne (1952) reported that varying the time-dose relationship of cortisone and influenza virus in infected chick embryos may result in no significant change in survival time of the embryos and a greatly increased virus concentration or an increased survival time and very little increase in virus concentration.

A cold invironmental temperature (Scherr, 1952), whole liver (Ershoff, 1951), and somatotrophic hormone (Selye, 1951) have all been shown to antagonize the deleterious effects of cortisone on normal animals. An attempt to preferentially antagonize the deleterious effect of cortisone on candida-infected mice with somatotrophic hormone (STH) was successful to a certain extent under rather exacting conditions (Scherr, 1953b). If the preferential antagonism of STH is a valid conclusion to be drawn from Scherr's data, then it should be possible to demonstrate this effect with other biological systems. Accordingly, a study of the effect of cortisone and/or STH on vaccinia-infected embryonated eggs was undertaken.

MATERIALS AND METHODS

White Leghorn eggs were used in all experimental and control groups. All eggs were incubated at 37 C and at a relative humidity of 50% for 12 days prior to infection. On the 12th day of incubation the eggs were candled to determine viability and to mark the areas of the chorioallantoic membrane and the air sac. These areas were then painted with merthiolate as an antiseptic precaution.

The technique used for drilling the eggs and inoculating the virus onto the chorioallantoic membrane was that described by Beveridge and Burnet (1946). The vaccinia virus suspension was prepared immediately before use by sus-

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pending the contents of one capillary tube (Smallpox vaccine, Sharp & Dohme) in 35 ml sterile physiological saline; 0.05 ml of this virus suspension constituted the infecting dose.

All eggs were treated immediately after infection. Parts of the shell over the chorioallantoic membrane were broken away and the underlying shell membrane was removed, thus exposing the yolk sac. The desired solutions were administered to the yolk sac by passing through the chorioallantoic membrane with a 20 gauge needle and tuberculin syringe.

Cortisone (Cortone ® acetate, Merck) suspended in Aqueous Vehicle No. 1 (Merck) was diluted so that the final concentrations were: 12.5 mg/ml cortisone, 0.7% benzyl alcohol, 0.7% sodium chloride, 0.45% polyoxyethylene sorbitan mono-oleate (Tween 80), and 0.5% sodium carboxymethylcellulose.

Somatotrophic hormone (Somatrofin ®, Frank W. Horner, Ltd., Montreal) was suspended in Aqueous Vehicle No. 1 to a final concentration of 6.25 mg/ml; the other ingredients of the aqueous vehicle remained the same as in the cortisone suspension.

A mixture of cortisone and STH in Aqueous Vehicle No. 1 was prepared so that the final concentrations for the components were equal to those of the cortisone, STH, and vehicle components described above.

Aqueous Vehicle was diluted with distilled water so that the final concentrations of the ingredients were equal to those in the above suspensions.

Five groups of infected eggs, approximately 25 eggs per group, were injected via the yolk sac as follows: (1) 0.2 ml physiological saline; (2) 0.2 ml cortisone suspension (2.5 mg cortisone per egg); (3) 0.2 ml cortisone and STH suspension (2.5 mg and 1.25 mg, respectively); (4) 0.2 ml STH suspension (1.25 mg STH per egg); (5) 0.2 ml aqueous vehicle. Uninfected eggs were injected in a manner similar to groups (2), (3), (4), and (5).

After injection the opening of each egg was sealed with transparent tape and the egg incubated for 3 more days. All the eggs were then opened and examined for viability, and a count was made of the lesions on the chorioallantoic membranes of viable eggs. Because the vaccinia lesions varied markedly in size as well as number,* each group of lesions per egg was arbitrarily graded according to the following scheme:

+, very small lesions
++, medium sized lesions
+++, large lesions
++++, very large lesions

Figures 1 to 4 illustrate the designations assigned to the lesions on typical chorioallantoic membranes. In considering both size and number each membrane was given a corrected number of lesions. Thus, a membrane with 10 lesions designated ++++ would have a corrected number of lesions of 40.

Eggs in which the embryo was dead due to technical mishap while handling or inoculating are not included in these data.

Where statistical comparisons of the corrected mean numbers of lesions were indicated, the method of t was employed; a P value of 0.05 or smaller is indicative of a statistically valid difference in averaged data between any two groups of eggs.

Tables 1 and 2 contain the results of this experiment. Mean corrected dissemination values for the various groups of eggs were computed with surviving embryos only.

The apparent protective effect of cortisone (8% mortality) against the death of the embryos due to the aqueous vehicle (35% mortality) was reversed when STH and cortisone were used together (33% mortality). STH used alone did not significantly alter this mortality rate (36%). STH did not significantly increase the number and size of vaccinia lesions (mean value of 48.6 as compared to 30.6 for infected controls treated with saline).

The aqueous vehicle did not appear to increase the number and size of virus lesions but did increase the mortality of the eggs. Infected embryos treated with aqueous vehicle showed essentially the same mortality rate as uninfected embryos similarly treated.

The effect of cortisone was to protect the embryos from the deleterious effect of the aqueous vehicle, as evidenced by a reduced mortality rate of 8%. STH reversed this protective action of

* It is generally conceded (Beveridge and Burnet, 1946) that, as with bacterial colonies, a single viral lesion arises from a single virus particle or clump of particles. One would expect, then, that all lesions would be similar in size if similar conditions were maintained. However, if the susceptibility of the cell is altered in favor of virus replication, the lesion size will be larger because of a facilitated dissemination of the virus from cell to cell. Since this work concerns itself not only with the ability of a given virus particle to invade a given cell, but also with the receptiveness of the cell to invasion, it was felt necessary to include in the experimental data a lesion size value as well as a lesion count value

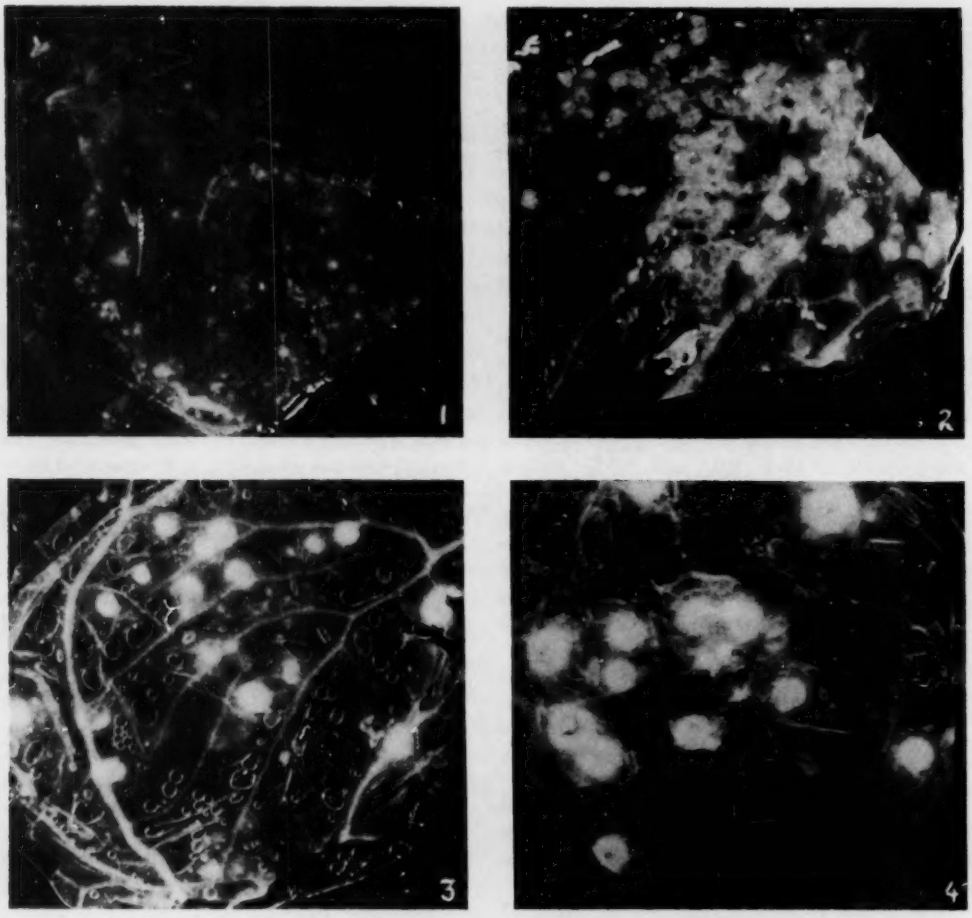

FIG. 1–4.—These figures represent the relative sizes of lesions found on the chorioallantoic membranes of eggs infected with vaccinia virus and treated with cortisone, somatotrophic hormone, or aqueous vehicle of cortisone. These lesions are graded as: figure 1, very small lesions (+), figure 2, medium sized (++), figure 3, large (+++), figure 4, very large (++++).

cortisone, since the mortality of eggs treated with cortisone and STH was comparable to that of eggs treated with the aqueous vehicle. However, eggs treated with cortisone and STH displayed a striking decrease in the number and size of viral lesions.

The experiment discussed here, with certain variations, has been repeated four times. It has been found that, depending on the size of inoculum, type of aqueous vehicle, and possibly other factors little understood, cortisone may increase or decrease the number and size of vaccinia lesions. However, it is significant that the lesions of eggs treated with cortisone and STH were always fewer and smaller than those in any other group. On one occasion the group of eggs treated with the mixture of cortisone and STH was the only one free of lesions.

DISCUSSION

We interpret the above data as indicating that the vehicle in which the cortisone is suspended is toxic to normal and vaccinia-infected embryonated eggs under the conditions of our experiments, as manifested by a mortality in both groups of approximately 35%. The mortality of eggs treated with aqueous vehicle must be attributed to the toxic effect of the benzyl alcohol component of the vehicle, since we have determined on a number of occasions that normal eggs inoculated via the yolk sac with 0.7% benzyl alcohol in distilled water

TABLE 1.—*The effect of inoculations into the yolk sac of cortisone and STH on vaccinia virus inoculated onto the chorioallantoic membrane of the embryonated egg.**

		I Infected, saline inoculated	II Infected, cortisone inoculated	III Infected, cortisone and STH inoculated	IV Infected, STH inoculated	V Infected, aqueous vehicle inoculated
Computation of mean dissemination values	Mean	30.6	21.3	5.2	48.6	32.7
	Average deviation	20.8	15.8	3.6	17.8	19.5
	Standard deviation	24.8	21.2	4.2	21.4	23.6
	Dead embryos/total	1/24	2/25	8/24	8/22	9/26
	Percent dead	4	8	33	36	35
	t (comparing groups IV and V)			1.89		
	P			0.1 – 0.05		
	t (comparing groups II and V)			1.56		
	P			0.2 –0.1		
	t (comparing groups II and III)			2.92		
	P			0.01–.001		
	t (comparing groups I and IV)			2.19		
	P			0.05–0.02		

* See text for dosage schedules and amounts.

or 0.7% benzyl alcohol in the Merck vehicle show comparable mortality rates (usually 25% to 30%). This toxic effect of the vehicle is almost completely reversed by the cortisone. In support of these contentions note that the mortality of infected and normal embryos treated with STH suspended in this vehicle is comparable to that of the groups treated with the vehicle alone (tables 1 and 2). Both the infected and uninfected groups of eggs inoculated with cortisone suspended in this vehicle showed a significantly reduced mortality rate; this reduction was reversed by STH. In addition, it has been possible to demonstrate a similar toxic effect of the vehicle in mice infected with moniliasis (Scherr, 1953a).

The reduction in number and size of viral lesions in eggs treated with the mixture of cortisone and STH is further emphasized if we score the number of eggs in each group against the relative size of lesions (table 3). It is obvious that in group III the distribution curve for size of lesions is heavily shifted toward smaller size or lack of lesions, whereas all the other groups have comparable distribution curves.

Our results reveal that two separate effects were taking place simultaneously and probably independently,—the toxic effect of the aqueous vehicle which was reversed by cortisone and the decrease in the number and size of viral lesions when cortisone and STH were used together.

Under the conditions of these experiments, cortisone or STH did not significantly effect the number and size of vaccinia lesions.

TABLE 2.—*The effect of inoculations into the yolk sac of cortisone and STH for normal embryonated eggs.**

	Cortisone inoculated	Cortisone and STH inoculated	STH inoculated	Aqueous vehicle inoculated
Dead embryos/total	3/27	12/29	6/26	9/27
Percent dead	11	41	23	33

* See text for dosage schedules and amounts.

TABLE 3.—*Number of eggs in groups.**

Size of lesions	I	II	III	IV	V
0	3	5	13	6	6
+	4	2	7	0	3
++	11	7	3	7	6
+++	6	9	1	5	9
++++	0	1	0	4	1
Percent chance that difference compared to group I is significant		31.1	99.99	44.5	1.6

* See table 1 for groups legends.

SUMMARY

Embryonated eggs infected with vaccinia virus on the chorioallantoic membrane and treated with cortisone showed a reduced mortality rate, but no significant change in number and size of lesions, as compared to infected eggs treated with the aqueous vehicle of cortisone containing benzyl alcohol. The concomitant use of cortisone and somatotrophic hormone reversed this protective effect of cortisone, but it significantly reduced the number and size of lesions as compared to eggs infected but untreated or those infected and treated solely with cortisone, somatotrophic hormone, or aqueous vehicle of cortisone.

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TRANSOVARIAN PASSAGE OF ANTIBODIES FOLLOWING NATURALLY ACQUIRED ENCEPHALITIS INFECTION IN BIRDS

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In the summers of 1950 and 1951 in Weld County, Colorado, collections of serums from nestling wild birds were made to obtain evidence of the extent of infection of these birds with western equine encephalitis (WEE) and St. Louis encephalitis (SLE) viruses. These serums were later tested for neutralizing antibodies against these viruses. In the course of the survey it was discovered that some nestlings, only one or two weeks old, had quite high neutralizing antibody indexes against one or the other of these viruses. These findings were interpreted as meaning that these high indexes were either the result of infection with the corresponding virus during the first few days after hatching, or that the antibodies had been acquired by the offspring through passive transfer from an immune mother by way of the egg.

Subsequent investigations to test the transovarian hypothesis revealed that a number of pigeon eggs collected in the study area contained a substance capable of neutralizing WEE and SLE viruses and that, in addition, some of the embryonic birds as well as others immediately after hatching possessed this neutralizing substance. This report records these findings and discusses their significance.

That antibodies engendered against a number of infectious agents in a bird subsequently can be transmitted to the offspring through the egg has been amply demonstrated. Among these are Newcastle disease virus antibodies,[1,2] diphtheria antitoxin,[3] antibodies to Rous sarcoma,[4] *Salmonella pullorum*, *Salmonella gallinarum*, and *Salmonella typhi*,[5] the transference of antibodies against infectious bronchitis,[6,7] and fowl plague.[1]

METHODS

A number of nestling birds were bled at weekly intervals in an attempt to isolate virus as well as to obtain information as to the presence and duration of neutralizing antibodies acquired through the egg. In addition, search was made for the appearance of antibody responses in young birds to serve as an indication of WEE or SLE infection acquired from an external source.

Eggs were collected from nests, the yolks were removed from non-embryonated eggs, ground with sterile alundum, and sufficient 0.2% bovine albumin was added to make a 50% suspension. This suspension was centrifuged at 1000 r.p.m. for 10 minutes. Antibiotics were added to the supernate, which was used in the neutralization tests without heat inactivation.

From embryonated eggs the embryos were removed, the heads discarded, and the remainder of the embryos ground, and a 50% suspension was prepared using 0.2% bovine albumin in buffered saline as a diluent. This suspension was centrifuged, antibiotics added, and these extracts were then tested for neutralizing properties against the WEE and SLE viruses.

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TABLE 1.—*Serums from nestling birds tested for neutralizing agents against WEE and SLE viruses.*

Species	WEE		SLE	
	Number tested	Number positive*	Number tested	Number positive
Common pigeon (*Columbia livia*)	687	79	108	4
Magpie (*Pica hudsonia*)	105	0	15	0
Red-winged blackbiard (*Agelaius phoeniceus*)	31	0	1	0
Yellow-headed blackbird (*Canthocephalus xanthocephalus*)	14	0	0	0
English sparrow (*Passer d. domesticus*)	89	2	9	0
Cliff swallow (*Petrochelidon a. albifrons*)	35	0	0	0
Barn swallow (*Hirundo rustica*)	10	0	0	0
Crow (*Corvus brachyrhynchos*)	1	0	0	0
Blue heron (*Ardea herodias*)	1	1	1	0

* Serums having neutralizing index of 32 or higher.

Neutralization indexes were determined by mixing undiluted serum, egg or embryo suspensions with tenfold serial dilutions of virus to determine the number of LD_{50} mouse doses of viruses inactivated by these materials, as compared to control titrations of the same virus inoculated intracerebrally into groups of 4 to 6 mice (CFW strain) weighing 12 to 15 g. Material with neutralization capacities of 32 LD_{50}'s (index 32) or higher were considered positive.

EXPERIMENTAL

The results obtained are presented in tables 1 through 4.

The immature birds tested for neutralizing antibodies are listed in table 1. Three of nine different species tested had antibodies against WEE, and there was one species (pigeon) with SLE antibodies. Serums were tested for SLE virus antibodies only during the 1950 season, at which time the ages of the small number of nestlings demonstrating antibodies ranges from 8 to 22 days.

Comparison of results of various weekly age groups of nestlings showed no appreciable differences in percentages of positives (table 2). Consequently, it would be difficult to deduce which in this group acquired antibodies by transovarian transfer and which by subsequent infection. It may be of interest to note that circulating WEE virus was demonstrated in two of nine species of nestlings tested in 1950[8] and five of the species in 1951.[9] Adult pigeons showed a comparatively higher incidence of antibodies which could very probably have resulted from virus infections (table 3). None of the adult birds had SLE antibodies.

Eggs and embryos tested in the areas where nestling pigeon serums had neutralizing antibodies showed a greater incidence of antibodies than might have been expected in a normal population sample (table 4). The number of egg samples is too small to make any comparisons with the number of nestling serums.

These findings obtained under natural conditions prompted additional studies

8. Sooter, C. A., Howitt, B. F., Gorrie, R. and Cockburn, T. A. 1951, Proc. Soc. Exper. Biol. & Med. **77:** 393–394.
9. Unpublished data.

TABLE 2.—*Nestling pigeon serums tested against WEE and SLE viruses. Antibody distribution by age groups.*

Age in days	WEE			SLE		
	Number tested	Number positive*	Percent positive	Number tested	Number positive	Percent positive
1–7	68	8	11.8	2	0	0
8–14	211	25	11.9	13	2	15.4
15–21	191	19	10.0	40	0	0
22–28	167	22	13.2	41	2	4.7
29 and over	50	5	10.0	12	0	0

* Serums having neutralizing indexes of 32 or higher.

TABLE 3.—*Adult bird serums tested against WEE and SLE viruses.*

Species		WEE		SLE	
		Number tested	Number positive	Number tested	Number positive
Mallard duck	*Anas p. platyrhynchos*	1	0	1	0
Marsh hawk	*Circus hudsonius*	1	1	0	0
Pheasant	*Phasianus colchicus torquatus*	3	0	1	0
Franklin gull	*Larus pipixcan*	1	0	1	0
Common pigeon	*Columba livia*	88	18†	14	0
Mourning dove	*Zenaidura macroura marginella*	11	1	0	0
Screech owl	*Otus asio*	1	0	1	0
Flicker	*Colaptes* spp.	2	0	2	0
Cliff swallow	*Petrochelidon a. albifrons*	3	0*	1	0
Blue jay	*Cyanocitta cristata*	1	0	2	0
Magpie	*Pica p. hudsonia*	2	1	0	0
Eastern crow	*Corvus brachyrhynchos*	1	0	0	0
English sparrow	*Passer d. domesticus*	21	3	11	0
Red-winged blackbird	*Agelaius phoeniceus*	11	0	0	0
Vesper sparrow	*Pooecetes gramineus*	1	0	0	0
	Total	148	24	34	0

Neutralizing indices of 32 and greater were considered positive. Neutralizing indices of 18 to 31 were considered equivocal.
* One cliff swallow was equivocal.
† One other pigeon was equivocal.

under experimentally controlled conditions. The results which were obtained and are reported in the two following papers[10,11] substantiate essentially all of the observations reported here, and extend them in certain respects.

DISCUSSION

The above findings are of significance in a number of respects. Embryonated hens' eggs are used in the laboratory frequently for the isolation or subsequent passage of certain neurotropic viruses. If flocks from which such eggs originate are exposed to a virus and antibodies against it are transferred to the egg, the chances of isolating that virus in such eggs would be diminished considerably.

The presence of neutralizing antibodies in bird serums has served as an indicator of past infection and as evidence of the current existence of the virus in the serums studied. If the neutralizing antibodies in the serums, particularly of young birds, is due to passive transfer from mother birds possibly infected in other areas, the data based on neutralization tests would have to be interpreted accordingly. Moreover, the existence of passively acquired antibodies among young birds would probably influence the ecology of the virus among such bird populations in a different manner than in birds without antibody.

TABLE 4.—*Pigeon eggs tested for neutralizing agents against WEE and SLE viruses.*

	WEE			SLE		
	Number tested	Number positive	Index	Number tested	Number positive	Index
Yolk	7	2	340 75	7	0	
Embryo	5	5	502 160 50 400 340	5	1	>50

SUMMARY

The serums of nestling birds obtained in their natural habitat were found to contain antibodies against western equine encephalitis and St. Louis encephalitis virus. That this resulted from passive transfer of maternal antibodies was indicated by the demonstration of similar antibodies in the egg yolks and embryos of birds of the same area. The significance of these findings is discussed.

10. Reeves, W. C., Sturgeon, J. M., French, E. M., and Brookman, B. 1954, J. Infect. Dis. **95**: 168–178.
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TRANSOVARIAN TRANSMISSION OF NEUTRALIZING SUBSTANCES TO WESTERN EQUINE AND ST. LOUIS ENCEPHALITIS VIRUSES BY AVIAN HOSTS

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The possibility that neutralizing antibodies to western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) viruses may be transmitted transovarially by immune female birds to their progeny was first advanced in 1951.[1] If transmission of this type commonly occurs it is obviously important that it be taken into consideration in interpreting the significance of the distribution of neutralizing antibodies to these viruses observed in birds of different ages in epidemiologic field surveys. It is also of significance in understanding the role young birds might play as hosts to these viruses.

Field observations and their implications, together with published data,[2] have led to the following additional laboratory and field studies in a different area and with other avian species. Since a dove colony was abailable at the Encephalitis Field Station in Kern County, California, it seemed appropriate to determine if substances protective against WEE and SLE viruses were transmitted transovarially to offspring doves and, if so, to know their duration. Of further interest was the response to virus infection of young birds to whom this antibody had been transmitted. A similar series of experiments were carried out with domestic chickens. In a third experiment a study was made of the virus susceptibility of embryonated eggs from a flock of domestic chickens naturally infected and immunized to WEE virus. Data gathered in these laboratory experiments were augmented by tests on wild birds from an endemic area.

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1. Sooter, C. A. and Schaeffer, M. 1951, Personal communication.
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METHODS AND PROCEDURES

Laboratory investigations

Virus strains.—The BFS 1034 strain of WEE and the BFS 2131 strain of SLE were originally isolated from mites collected in Kern County, California, and each had undergone 4 mouse brain passages after its original isolation.

The F103 strain of SLE virus was first isolated from mosquitoes collected in the Yakima Valley, Washington. The actual number of mouse brain passages is unknown, but it had undoubtedly been more than 4. The Olitsky strain of WEE virus and the Webster strain of SLE virus were used in testing all of the bird serums by mouse neutralization test.

Stock ampoules of 20% mouse brain suspension of virus diluted with a solution consisting of 50% rabbit serum and 50% beef broth were prepared and frozen until used. Mouse titrations were run periodically on a sample ampoule of virus to determine whether the titer had dropped during storage.

Mice.—Mice used in the neutralization and viremia tests were from a colony of inbred white Swiss mice maintained at the Hooper Foundation. Original breeding stock was obtained from the Rockefeller Institute strain. Mice used in the neutralization or viremia tests were 21 days old

Parent birds and their immunization.—A "mosquito-proof" aviary was used to isolate the parent doves and chickens for the transovarian studies. Four pairs of hybrid doves (*Streptopelia* spp.) were placed in cages in pairs. Three laying hens and a rooster of white Leghorn stock were placed in one section of the aviary. Blood samples were drawn from all the birds and tested by mouse neutralization test to determine the pre-inoculation levels of antibody against WEE and SLE viruses.

The subcutaneous route of inoculation of virus was selected for all the birds. The dilution of the inoculum was selected from results of intracerebral mouse titrations of each virus strain used and is given as such in all references to dilution of virus. It must be remembered that in all instances the actual mouse LD_{50} of virus in the inoculum used in the birds is considerably greater (6.6 to 8.3 times the mouse inoculum) due to the difference in quantity of dose—0.2 to 0.25 ml in the birds, 0.03 ml in mice.

The original plans called for a single inoculation of virus for each bird, but additional inoculations were found to be necessary to produce high antibody levels in the 4 parent doves in the experiments with SLE virus. The neutralization indexes of serums from 4 doves inoculated with WEE virus were high after inoculation. The 3 hens were given 2 inoculations, 1 of WEE virus and 2 weeks later 1 of SLE virus. The immunization schedule of the parent birds is given in table 1.

Progeny.—Each pair of parent doves produced offspring every 5 to 6 weeks during the spring and summer, and usually there were 2 young each time. They produced offspring with great regularity throughout the spring and early summer and again in the winter, with a lull in egg production during the fall. A total of 28 offspring was hatched during the 54 weeks of the study.

Blood (2.0 ml) was taken from a wing vein of each young bird 2 weeks after it hatched, and selected birds were bled again at 4, 8, 12 and 16 weeks of age. Serums from these samples were tested to determine the neutralization index with the virus with which the parents had been inoculated. Young self-sufficient birds being held for further study were removed from the parents and liberated into the aviary, usually when they were 25 days old.

TABLE 1.—*Immunization schedule for parent doves and chickens.*

Birds		Virus inoculum			
		Amount, ml	Mouse LD_{50}'s	Strain	Type
Doves	2 pairs	0.25	100	BFS 1034	WEE
Doves	2 pairs	0.25	100	BFS 2131	SLE
		0.2	1000	BFS 2131	
		0.2	100	F 103	
Chickens	3 hens	0.2	100	BFS 1034	WEE
		0.2	100	F 103	SLE

Eggs from the chickens were collected daily without regard to parentage and were hatched in an incubator. The chicks were kept inside the field laboratory in screened rooms. A blood sample (1 ml) was collected from the heart of each chick at 2 weeks of age, and the serums were tested to determine neutralizing antibody levels to both WEE and SLE viruses. Further blood samples for the neutralization test were taken from selected chicks 4, 8 and 12 weeks of age.

Inoculation tests for viremia and antibody levels.—Eight progeny of WEE-inoculated doves were held until mouse neutralization tests showed their serums to be lacking in antibody. Each dove was then given a subcutaneous inoculation of 0.2 ml of a 100 mouse LD_{50} dilution of BFS 1034 WEE virus. Blood samples were drawn 24 and 48 hours after inoculation, and the serums were placed in ampoules and frozen immediately. To learn whether or not the virus was present in the blood of these doves, a portion of each serum sample was injected into 5 mice (0.03 ml intracerebrally and 0.09 ml intraperitoneally). If any of these mice had symptoms of encephalitis, its brain was removed and used for passage into 5 additional mice. If 2 or more of these 5 mice died after having had central nervous system signs of encephalitis, it was assumed that virus had been present in the blood of the dove.

Additional serum samples were drawn from the doves 2, 4 and 8 weeks after virus inoculation and were tested for neutralizing antibody.

A similar study could not be made with SLE virus in doves because of the poor infecting or immunizing qualities of the virus strains being used.

The serums of 8 offspring chicks were tested at 2- to 4-week intervals until the neutralization test indicated that the antibodies transmitted by the hen had disappeared. Each chick was then given a single subcutaneous inoculation of BFS 1034 strain of WEE virus—0.2 ml of 100 mouse LD_{50} dilution. Blood samples were taken 24 and 48 hours after inoculation, and virus tests were made by the procedure used on the dove serums. Serum samples were also taken 2, 4, 8 and in some cases 12 weeks after inoculation and used in the neutralization test.

Five additional offspring chicks that had lost their maternal antibody were inoculated with 0.2 ml of 100 mouse LD_{50} of F103 strain of SLE virus by the subcutaneous route. Blood samples were taken 24 and 48 hours after inoculation to learn whether the virus was present in the blood,

and 2, 4 and 8 weeks after inoculation to determine the neutralizing antibody level.

An additional 14 chicks from immunized hens were bled at 2 weeks of age when maternal antibodies were expected to be present. They were then immediately inoculated subcutaneously with 0.2 ml of 100 mouse LD_{50} concentration of WEE virus (BFS 1034 strain). Tests of serums taken on the date of inoculation showed a range in protective antibody levels from almost negative to several logs by mouse neutralization test. Only one postinoculation blood sample was taken, 3 weeks later, and tested for WEE antibody.

Control birds.—Several settings of eggs from WEE-inoculated doves were moved to a cage with SLE-inoculated parents for incubation and rearing, and eggs of SLE-inoculated doves were hatched and the young were raised by WEE-inoculated doves. This was done in an effort to learn whether offspring might be infected by contact with adult birds. Parasitic mites were observed on the immature doves for only a short time during these experiments and were successfully eliminated by intensive insecticide treatment of the aviary.

Blood samples were taken from 24 offspring of uninoculated doves in a separate colony. These were bled 2 weeks after hatching and served as a control for the possible occurrence of nonspecific neutralizing substances in immature birds. The offspring of SLE-inoculated doves hatched prior to the third inoculation also served as normal control birds.

Ten chicks hatched from eggs laid prior to inoculation of the parents were used as the normal controls for the chicken studies.

Titration of WEE virus in embryos.—Twenty hens (Rhode Island Red or Hampshire Red) with WEE neutralization indexes of more than 100 (range: 146 to 420) were selected from a farm flock and placed with 2 roosters in an outdoor pen in Bakersfield. Eggs were collected daily, and 60 were shipped to the San Francisco laboratory for incubation. For controls, fertile eggs were procured from sources in the San Francisco Bay area, a nonendemic area for the WEE virus: presumably these eggs were from chickens that had not been exposed to this virus.

A comparative susceptibility titration to WEE virus, F199 strain, was made in the test and control eggs. The amniotic cavity of each 10-day-old embryo was inoculated with 0.1 ml of virus suspension. Serial tenfold dilutions of virus were tested, covering the range of 1000 to 0.001 mouse LD_{50}'s. An embryo inoculated with 0.1 ml of the 1000 LD_{50} dilution received the equivalent of 3333 mouse LD_{50}'s. Virus dilutions were retitrated in mice at the same time, using 5 mice for each tenfold dilution, 1000 LD_{50}'s through 0.001 LD_{50}, and the mouse LD_{50} was recalculated. The inoculated embryos were placed in a 35 C incubator and were candled several times a day to learn the time of the deaths. Death of the embryo within 16 hours after inoculation was considered nonspecific, due to trauma.

Embryos surviving for 7 days were incubated for the usual 21-day hatching period. Chicks that hatched were kept in an electric brooder until they were 2 weeks old, when samples of blood were taken for antibody tests.

Neutralization test and selection of significant indexes.—In the mouse neutralization tests, undiluted serums were tested against 10 and 100 LD_{50} concentrations of WEE or SLE viruses (also 1000 to 10000 LD_{50}'s if there was enough serum).

The serum being tested (0.15 ml) was mixed with an equal amount of the test virus dilutions (previously calculated by the Reed and Muench method for LD_{50}[3] and allowed to incubate in a 37 C water bath for 2 hours. A control titration of the virus dilutions was run simultaneously, using normal rabbit serum, inactivated at 56 C for 30 minutes, in place of the test serums. Each serum-virus suspension (0.03 ml) was inoculated intracranially into each of 4 mice, and deaths of mice occurring after the appropriate incubation time for the virus strain used were recorded. The neutralization index of each serum was calculated after the LD_{50} of the virus dilutions had been determined from the normal rabbit serum controls.

The results of past surveys of serums from wild and domestic birds with naturally acquired and laboratory-induced infections led us to accept a neutralization index of 50 or more in an inoculated bird as representative of a positive result. It was more difficult to determine a neutralization index representative of a positive result in serums from offspring tested for maternally transferred antibody. The level of transmitted antibody was expected to be lower than that in the parents.[4,5]

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4. Brandly, C. A., Moses, H. E. and Jungherr, E. L. 1946, Transmission of antiviral activity via the egg and the role of congenital passive immunity to Newcastle disease in chickens. Am. J. Vet. Res. **7**: 333–342.
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Blood from control doves and chicks never had any detectable neutralization index to WEE or SLE viruses. An arbitrary positive index of 20 was finally chosen on the basis of findings in the 26 offspring of WEE-inoculated doves: neutralization indexes were 20 or greater in at least two-thirds of the serums taken 2 weeks after these birds had hatched.

The first 3 positive serums taken from 2-week-old offspring of WEE-inoculated doves were heat-inactivated to determine what effect this might have on the antibody. The 3 serums were tested after inactivation of 56 C for 20 minutes and also without inactivation. Two of the three serums maintained comparable levels of protection after inactivation, but the index of the third dropped to an insignificant level. Serums were not inactivated with heat in any of the later studies.

Field investigations

Blood samples were collected from trapped or shot specimens of the common house finch (*Carpodacus mexicanus frontalis*) and the western mourning dove (*Zenaidura macroura marginella*), in two highly endemic study areas in Kern County. The birds were divided into three age groups: adults, immature birds between 1 and 6 months of age and nestling-fledglings less than 1 month of age. Serums from these birds were tested for neutralizing antibodies by the procedure already described. A neutralizing index of 50 or more in an adult bird and an index of 20 or more in the younger two classes was accepted to represent a significant antibody level Most of the seropositive young birds had indexes above 50.

RESULTS

Laboratory studies

WEE virus antibodies in doves.—Adults.—The 4 parent doves had high neutralization indexes after one inoculation of virus (table 2).

Progeny.—The total number of doves hatched was 28. Nineteen of the 25 birds tested at 2 weeks of age (table 2) had a neutralization index to WEE virus of 20 or greater (76% positive). The index ranged from 24 to 1540. They were highest (500 or more) in birds hatched during the first 16 weeks after inoculation of the parents. Eight of the 14 serums from 4-week-old birds showed positive neutralization indexes (57.1% positive). These birds had hatched between the 10th and 27th weeks after inoculation of the parents. Of 8 doves tested at 8 weeks of age only 2 had positive indexes (25% positive). Serum from 7 birds 12 weeks after hatching included 2 with positive antibody levels (28.6% positive). One dove (B4272) which had been seronegative at 8 weeks now had a detectable titer. Of the 2 doves with a significant index at 8 weeks, in one it had dropped below 20 and in the other it remained above 20. All 7 birds tested at 16 weeks of age were completely lacking protective substances to WEE virus; 5 had been positive at an earlier age.

Susceptibility to infection.—Eight young doves were inoculated with the BFS 1034 strain of WEE virus after the loss of maternal antibodies (table 3). Three of these birds had viremia 24 or 48 hours later. Two weeks after inoculation 6 of the 8 had a neutralization index of 50 or more. Three of the 6 positive serums came from birds previously shown by the viremia tests to be infected. Four weeks after inoculation 2 of the 3 birds tested that had been seropositive earlier still had significant antibody levels. At the last bleeding, 8 weeks after inoculation, 5 of 6 birds tested had positive indexes. The index of the serum from bird B4271 was slightly higher than it had been, but it still remained below 50.

SLE virus antibodies in doves.—Adults.—Difficulty was encountered in producing antibody titers in the parent doves with the BFS 2131 strain (table 4), even after a second inoculation of 1000 mouse LD_{50}'s. The third inoculation with the F103 strain produced a sufficiently high index in one female so that progeny subsequently hatched

hemagglutination inhibition titers in baby chicks to their actual immunity. Am. J. Vet. Res. **8**: 379–382.

TABLE 2.—*Transovarian transmission of WEE neutralizing antibodies by doves (posthatching phase).*

Bird no.	Cage no.	Sex	Neutralization index			
			Before inoculation	After inoculation*		
				4 wk.	32 wk.	60 wk.
Parents						
D406	2	M	1	6650	715	299
D416	2	F	37	6650	1060	109
44-404729	3	M	1	6650	157	299
42-401939	3	F	2250	3160	1570	>500
Offspring						
		Week†	Age, week			
			2	4	8	12	16
–B	3	4	1540				
–D	3	4	715				
–C	2	4	154				
–A	2	4	1				
B4255	3	10	1540	37			
B4256	3	10	715	37			
B4258	2	10	154	37	37	32	1
B4257	2	10	124	1			
B4269	3	15		1	1	1	1
B4270	3	15		1	1	1	1
B4272	2	16	539	24	1	24	1
B4271	2	16	118	188	36	12	1
B4290‡	3	21	271		1	1	1
B4291‡	3	21	59	1	1	1	1
B4300‡	2	23	1	1	1		
B4311	3	27	115	36			
B4312	3	27	115	36			
C4310	2	27	24	1			
C4309	2	27		18			
C4327‡	2	38	24				
C4328‡	2	43	15				
C4329	3	43	150				
C4330	3	43	70				
C4339	2	48	15				
C4340	2	48	15				
C4345	3	53	336				
C4350	2	54	139				
C4351	2	54	1				

* Subcutaneous inoculation of 0.25 ml of 100 mouse LD_{50}'s, BFS 1034 strain.
† Time between parent inoculation and hatching date of offspring.
‡ Control, eggs incubated and reared by SLE-inoculated parents.

had serum antibody levels comparable to those in the offspring of WEE-inoculated parents. The second female also produced some antibody against SLE virus, but the index was very low and the results of serologic examination of most of her progeny were negative.

Progeny.—The 7 offspring doves hatched from the pair of doves in cage 4 after the parents had been inoculated 3 times had neutralization indexes greater than 20 at 2 weeks of age (table 4). Among the 6 offspring of the doves in cage 1, after the third inoculation of the parents, there was only 1 bird with an index above 20 at 2 weeks of age. Only 5 birds were bled at 4 weeks of age—2 birds from cage 4 and 3 birds from cage 1. One of the 2 birds from cage 4 retained an antibody level. One of the 3 birds from cage 1 had a significant index, although it had not been significant when the same bird was tested at 2 weeks of age. None of these 5 birds were

TABLE 3.—*Response of doves inoculated with WEE virus after loss of maternal antibody.*

Bird no.*	Cage no.	Age at inoculation weeks	Viremia†	Neutralization index		
				Weeks after inoculation‡		
				2	4	8
B4258	2	24	0	1		
B4271	2	24	0	1	15	32
B4290	3	19	0	240	250	220
B4291	3	19	0	192	320	1240
B4269	3	19	+	115		150
B4270	3	19	+	53		1800
B4272	2	18	+	77		
B4300	2	11	0	77		565

* For previous history of these birds see table 1.
† Viremia present if mouse passage was successful from either 24 or 48 hour blood sample.
‡ Subcutaneous inoculation of 0.2 ml of 100 mouse LD_{50}'s, BFS 1034 strain.

TABLE 4.—*Transovarian transmission of SLE neutralizing antibodies by doves (posthatching phase).*

Bird no.	Cage no.	Sex	Neutralization index				
			Before inoculation	After inoculation*			
				No. 1	No. 2	No. 3	
				4 wk.	4 wk.	2 wk.	4 wk.
Parents							
D415	1	M	1	1	180	1240	1
44-404749	1	F	1	1	180	38	18
44-422552	4	M	1	15	8	8	18
D405	4	F	1	1	1	1240	55

Offspring				
Bird no.	Cage no.	Week†	Age, weeks	
			2	4
C4317	4	8	240	
C4315	1	8	24	
C4314	1	8	19	
C4344	4	32	320	
C4343	4	32	47	
C4361	4	38	257	
C4360	4	38	82	
C4359	1	38	1	
C4369	4	43	>55	
C4368	1	43	1	1
C4382	4	52	53	36
C4383	4	52		1
C4388	1	53	1	25
C4389	1	53	1	1

* Subcutaneous inoculation:
No. 1: 0.25 ml of 100 mouse LD_{50}'s ,BFS 2131 strain.
No. 2: 0.25 ml. of 1,000 mouse LD_{50}'s, BFS 2131 strain.
No. 3: 0.25 ml of 100 mouse LD_{50}'s, F103 strain.
† Time between the third inoculation of the parents and the hatching

tested further, nor were any of them inoculated with virus.

Control doves.—Five eggs from WEE-inoculated parents were hatched and the offspring were reared by SLE-inoculated doves, and 1 egg (laid prior to the third inoculation of the parent) from SLE-inoculated parents was hatched and the progeny was raised by WEE-inoculated birds. These 6 birds were bled at 2 weeks of age, and the serums were tested for neutralizing antibodies. In the 3 cases in which evidence of WEE virus antibodies was found in the offspring serums it was to the same virus as that found in the female dove that had laid the egg (table 2).

The 24 offspring hatched by 6 pairs of uninoculated parent doves were bled at 2 weeks of age. Antibodies were not found for either WEE or SLE viruses.

The first 13 progeny of the doves in cages 1 and 4, prior to the third inoculation of the parents, were all negative when tested 2, 4, 8, 12 and 16 weeks after hatching.

WEE virus antibodies in chickens.—Adults.—The neutralization indexes of 3 hens rose to and were sustained at a high level after 1 inoculation of virus (table 5).

Progeny.—The evidence of maternally transferred antibodies in offspring chickens was essentially similar to that in offspring doves, except that in the chicks antibodies were detectable for a shorter time (table 5). Thirty-seven chicks were bled at 2 weeks of age, and serums of 28 had significant antibody levels (75.8% positive). At 4 weeks of age 11 chicks were bled again, and the serums of only 2 were positive (18.2% positive). The same 11 chicks were again tested at 8 weeks of age, and by then the indexes of all had dropped to insignificant levels. Seven of these 11 chicks were tested again at 12 weeks, and there

TABLE 5.—*Transovarian transmission of neutralizing antibodies to WEE and SLE viruses by chickens (posthatching phase).*

Bird no.	Sex	Neutralization index								
		WEE virus					SLE virus†			
		Pre-inoculation	After inoculation,* wk				Pre-inoculation	After inoculation,† wk		
			4	17	40	56		2	15	38	54
343	F	1	66	>360	109	>270	57	360	78	380	376
344	F	1	710	>3600	749	>270	1	1	1	1	1
345	F	1	1150	>360	500	184	1	12	1	18	1
346	M	1	No inoculation			1	1	No inoculation			1

Offspring										
	Week‡	Age, weeks				Week‡	Age, weeks			
		2	4	8	12		2	4	8	12
C4220	3	168				1	115			
C4215	3	66				1	1			
C4219	3	55				1	1			
C4221	3	19				1	1			
C4217	3	12				1	24			
C4225	4	240				2	1			
C4228	4	115				2	1			
C4227	4	24				2	36			
C4224	4	12				2	1			
C4234	5	240	157	11	1	3	1	1	1	
C4235	5	66	1	1		3	1	1		
C4233	5	36	1	1		3	1	1	17	
C4229	5	19				3	1			
C4236	6	196	34	1	1	4	1	1	1	1
C4241	6	72	1	1	1	4	1	1	1	1
C4237	6	1	1	1		4	>50	1	1	
C4240	6	1	1	1		4	27	1	1	
C4243	6	1	1	1	1	4	16	1	1	1
C4248	7	490	1	1	1	5	16	1	1	1
C4250	7	157				5	1			
C4249	7	34	1	1	1	5	27	1	1	1
C4246	7	34	1	1	1	5	16	1	1	1
52-401	41	109				39	38			
52-402	43	9				41	1			
52-403	43	3								
52-413	46	336				44	1			
52-414	46	124				44	1			
52-418	46	124				44	1			
52-407	46	63								
52-410	46	63								
52-409	46	30								
52-421	47	336				45	1			
52-423	47	336				45	1			
52-422	47	242				45	1			
52-425	48	264				46	1			
52-427	49	264				47	1			
52-429	49	124				47	36			

* WEE, subcutaneous inoculation: 0.2 ml of 100 mouse LD_{50}'s, BFS 1034 strain.
† SLE, subcutaneous inoculation: 0.2 ml of 100 mouse LD_{50}'s F103 strain.
‡ Time between inoculation of parent and date the egg was laid.

were no indexes above 20.

Susceptibility to infection.—After the loss of maternal antibody, 8 chicks were inoculated with WEE virus BFS 1034 (table 6); of these, 6 had demonstrable viremia 24 or 48 hours later. Two weeks after inoculation, serums of 7 of the 8 had neutralization indexes above 50. Chick C4236 did not have a significant index despite its earlier viremia. The 2 birds that had not had demonstrable viremia had indexes above 50 at this bleeding. The indexes were higher than they had been at the 2-week bleedings made during the posthatching or maternal antibody phase. Five of 6 chicks tested 4 weeks after virus inoculation were seropositive. The index of chick C4236 was still not significant. The titer of serveral serums was higher than it had been. Five chicks tested 8 weeks after inoculation all had high indexes. The indexes of nearly all the birds rose between the 2nd and 9th weeks after inoculation, while in the same amount of time in the earlier posthatching phase indexes had dropped rapidly. Only 2 chicks were bled as late as 12 weeks

TABLE 6.—*Response of chicks inoculated with WEE virus after loss of maternal antibody.*

Bird no.*	Age at inoculation, weeks	Viremia†	Neutralization index				
			Before inoculation‡	After inoculation,§ weeks			
				2	4	8	12
C4234	16	0	1	>470	320	920	
C4248	16	+	1	>470	470		
C4249	16	+	1	>470	320	430	
C4241	16	+	1	150	220	430	
C4246	16	+	1	150	>470	>1390	
C4236	16	+	1	47	25	250	
C4233	8	+	1	320			>1390
C4235	8	0	1	151			>1390

* For previous history of these birds see table five.
† Viremia present if mouse passage of either 24 or 48 hour blood sample indicated infection.
‡ Level of transmitted antibody at the time of inoculation.
§ Subcutaneous inoculation: 0.2 ml of 100 mouse LD_{50}'s, BFS 1034 strain.

after inoculation, and both had high indexes.

Fourteen chicks were inoculated with virus at 2 weeks of age, before they had lost their maternal antibody. The indexes were then in the range of 63 to 336 in 11 of the 14 and were below 50 in the remaining 3. All of these chicks had an index of 1 when tested 3 weeks after virus inoculation.

SLE virus in chickens.—Adults.—Only 1 hen (no. 343) of the 3 inoculated developed neutralizing antibodies to SLE virus (table 5), and this undoubtedly accounts for the scarcity of seropositive chicks in this study.

Progeny.—Eight of the 33 serums tested from 2-week old chicks (table 5) had significant indexes (24.2% positive) None of the serum samples from chicks 4, 8 and 12 weeks of age had significant indexes. These samples included serum from only 3 chicks that had had indexes above 20 at the 2-week bleeding.

Susceptibility to infection.—Five of these 33 chicks were later inoculated with virus. The viremia tests were completely negative, and the indexes were 1. Apparently none of these birds was successfully infected with this virus.

Control chickens.—The only control birds were the 10 chicks hatched from eggs laid prior to virus inoculation of the hens. In the neutralization test of serums from the chicks, taken at 2 weeks of age, the results with WEE virus were all negative. The index of 1 of the 10 chicks was above 20 in the neutralization test with SLE virus. This chick may have been an offspring of hen 343 (table 5), which had a preinoculation serum titer to this virus.

WEE virus titrations in embryonated eggs.—Data on virus titrations in 10-day-old embryonated eggs from naturally immune and control chickens are summarized in table 7. In this single titration there appeared to be at least a 2 log difference in susceptibility, the eggs from immune chickens being mark-

TABLE 7.—*Comparative titration of WEE virus in 10-day-old chick embryos from immune and nonimmune parents and in mice.*

Log virus dilution inoculated	Precalculated number of mouse LD_{50}'s	Results of titration			
		Deaths/total inoculated			
		Eggs from immune hens*	Eggs from nonimmune hens*		Mice
			I	II	
4.7	1000	5/5	2/2		5/5
5.7	100	6/6	6/6		5/5
6.7	10	2/6	6/6		5/5
7.7	1	3/5	5/5	2/2	2/5
8.7	0.1	3/6	4/5	2/2	0/5
9.7	0.01	1/5	3/5	1/3	0/5
10.7	0.001	0/5	2/5	0/2	0/5
Saline control		0/5	1/3		
Calculated LD_{50} dilution†		7.9	10.0	9.5	7.5
Log difference from mouse titration		0.4	2.5	2.0	

* Immune hens in a Bakersfield flock; nonimmune hens I, from Poultry Science, Berkeley; nonimmune hens II, from Petaluma.
† 50% end point calculated by the Reed and Muench formula.

TABLE 8.—*Age distribution of neutralizing antibodies to WEE and SLE viruses in wild house finches and mourning doves.*

Bird	Age, months	WEE virus			SLE virus		
		Number tested	Number positive	Percent positive	Number tested	Number positive	Percent positive
House finch	>6	38	16	42	12	2	17
	1–6	24	0	0	6	0	0
	<1	17	3	18	12	4	33
Mourning dove	>6	20	2	10	19	8	42
	<1	44	1	2	43	13	30

edly less susceptible, as might be expected if their tissues contained neutralizing substances.

There was no apparent active antibody response in chicks hatched from embryos that survived the virus titrations. Five chicks from the naturally immune flock of hens had neutralizing indexes ranging from 24 to >360 at 14 days of age. The highest index was produced by one of the embryos inoculated as a control with saline as the inoculum. There was no correlation between the amount of virus used as inoculum in the embryo and the neutralization index of the chick at 14 days of age. One chick from a nonimumne source which survived virus titration in the embryo was completely lacking in serum neutralizing antibody at 14 days of age.

Field studies

Further support of the results of the experimental observations is offered by the distribution of neutralizing substances in a small group of common house finches and western mourning doves of different ages (table 8). At least some of the sero-positive birds under 1 month of age may have had maternally transmitted antibody.

DISCUSSION

In these studies of doves and chickens it was found that neutralizing antibodies to WEE and SLE viruses were transmitted by immunized parents to their progeny through the egg if antibody levels were sufficiently high in the parent. The protective substance in serums of newly hatched doves and chickens that were progeny of immunized parents was usually of a lower titer than that in the serums of the parents. This substance completely disappeared while the offspring were still quite young. The concept that this is a naturally occurring process is supported by the age distribution of antibodies in wild common house finches and western mourning doves exposed to infection and immunization in an endemic area. These findings are quite similar to those reported by other workers[4,6–15] with

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avian hosts and a wide range of other infections and immunizing agents.

Active immunization with WEE virus or, more truly, challenge inoculation by a peripheral route while the maternal antibody was present in the serum of the offspring did not lead to antibody production. This may indicate that the protective substance or passive immunity conferred on the offspring was sufficient to protect the bird from active infection, at least with peripheral inoculation of the virus dose used in this study.[16–21] Similar observations have been reported with other virus infections of birds[4,13,22,23] It is also possible that the phenomenon may be of the type described by Slavin,[24] in which mice passively immunized against SLE virus were successfully infected with the virus, and the infection apparently persisted in the central nervous system for up to 162 days. Still another possibility is that young birds lack any antibody-producing mechanism;[25] this in itself could account for the results obtained. However, there is no question that young doves can become infected with these viruses and develop neutralizing antibodies against them. In other experiments in this laboratory, 17 doves, 6 to 15 days of age, were infected with WEE virus and developed antibodies, and 15 doves, 7 to 15 days old, responded similarly to SLE virus inoculation. Only limited studies have been made in this laboratory on the immune response of young chicks after infection with these viruses. Chicks 2 to 6 weeks old developed anti-bodies after WEE virus infection, and chicks 4 to 8 weeks old developed SLE antibodies.

Such studies should be extended to include birds from unimmunized parents, virus inoculation of birds at different ages and tests for viremia as well as for antibody.

The possibility that virus transmission from parents to offspring was the stimulus to antibody production seems unlikely in view of the transience of the immmunity of the young birds, a characteristic of passive rather than active immunity.

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The possession of maternal antibodies by nestlings in the field should not rule them out as possible hosts of virus either as immature birds or as adults. No bird having lost its maternal antibody and then having viremia either 24 or 48 hours after virus inoculation failed to respond to active infection by formation of antibodies. However, in interpreting the results of surveys of the serum antibody levels of wild birds to determine whether they have been infected, the age of the tested birds should be taken into account.

The fact that substances capable of neutralizing WEE virus were transmitted through the fertile eggs of chickens should probably be of concern to laboratories using chick embryos as a routine tool in isolating these encephalitis viruses. Antibodies in the embryo seem to have a definite effect on the susceptibility of the embryo to virus infection.

SUMMARY

Domestic doves and chickens immunized with western equine or St. Louis encephalitis viruses transmitted neutralizing substances to these viruses through the egg to their offspring. The immunity conferred on the offspring was short, lasting a maximum of 12 weeks in doves and of 4 weeks in chickens, with a gradual decrease in the neutralization index from 2 weeks of age on. A proportion of young birds inoculated with WEE virus after loss of maternal antibody developed viremia within 48 hours—37.5% in doves and 75% in chickens. After loss of maternal antibody all the chickens and 83% of the doves inoculated with WEE virus had high neutralization indexes and maintained them until termination of the experiment 8 to 12 weeks later. There was no evidence of infection after inoculation of WEE virus into young birds with maternal antibodies. No chick inoculated with SLE virus after loss of maternal antibody produced antibodies to the virus, but there was no evidence of viremia or successful infection. The distribution of antibodies in different small age groups of wild birds is further evidence of the transovarian passage of neutralizing antibodies. Ten-day-old embryonated eggs from immune chickens were markedly resistant to WEE virus infection.

TRANSFER OF MATERNAL NEUTRALIZING ANTIBODIES AGAINST EASTERN EQUINE ENCEPHALOMYELITIS VIRUS IN BIRDS

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During the course of investigations into the ecology of eastern equine encephalomyelitis (EEE) in the southern United States, bloods have been collected from young and adult birds and then tested for virus or antibody. A rookery of white ibis (*Guara alba*) has received somewhat detailed attention in this respect; and on the several occasions that this rookery was sampled, it was found that neutralizing antibodies were present in the nestling as well as in the adult birds.

No virus was recovered from any of the specimens of ibis serum collected over the 3-year period ending in 1952.

Because of an interest in the chronology of the infection in nature, the question arose as to whether the antibody observed in the young ibis was the result of infection or was of maternal origin. That the latter is possible has been shown by others, who demonstrated that certain antibodies can be transferred from hens to their progeny.[1]

METHODS

Virus.—A strain of EEE virus isolated from *Culiseta melanura* was used.[2] It had received two mouse brain passages and one chick embryo passage. Virus in this last passage was used for both inoculation of birds and in the neutralization tests.

Pigeons.—Adult domestic pigeons (*Columba livia*) showing no neutralizing antibody to EEE were used. Those birds surviving subcutaneous inoculation of virus were placed in a wire-enclosed outdoor aviary and allowed to breed. The young birds hatching from eggs laid by the recovered adults were bled at periodic intervals, the blood being diluted 1:10 in buffered saline and the cells removed by centrifugation. Previous tests on chicken serum showed that neutralization was of the same order whether undiluted serum or a single tenfold dilution was used.

Titrations of virus and antibody.—All titrations of virus were made by intracerebral inoculation of mice with tenfold dilutions of the suspected material, using 4 mice per dilution. The LD_{50} was calculated by the method of Reed and Muench[3] and is based upon volumes of 0.03 ml. Neutralization tests were performed by mixing serial tenfold dilutions of virus with undiluted serum, or plasma diluted 1:10, and incubating overnight in the refrigerator before inoculating each mixture intracerebrally into 4 mice. The neutralization indexes are expressed as the antilog of the ratio of the LD_{50} end points of the normal control and of the unknown serums. An index of 30 or greater was considered as positive neutralization.

EXPERIMENTS

In 1950, 3 of 10 adult and 9 of 37 young (approximately 2 weeks old) white ibis had positive neutralizing indexes against EEE. In 1951, 19 young ibis (2 weeks old) were bled at the rookery and another 19 young birds of the same age were brought back to the laboratory and bled 4 weeks later. Three of 19 ibis bled at 2 weeks of age showed positive indexes, while only 1 of the 19 bled at 6 weeks of age had neutralizing antibodies to EEE. Fifty-six serum specimens from adults and 55 from nestlings were collected from the ibis rookery again in 1952. The nestlings were grouped according to approximately 1 to 1½ weeks of age and 2

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TABLE 1.—*Neutralizing antibody in the serums of white ibis.*

Year	Adult	1–1½ weeks	2–2½ weeks	6 weeks
1950	3/10*		9/37	
1951			3/19	1/19
1952	17/56	3/9	6/39	

* 3/10—3 of 10 serums tested showed neutralizing antibodies to EEE virus.

to 2½ weeks of age. Although one group is small, a higher incidence of neutralizing antibody in the younger group is indicated (table 1).

In spite of the limited numbers involved, it appeared that the observed antibody was being transferred through the egg, since specific neutralizing capacity appeared to diminish with age in these young birds. In addition, it was thought that failure of virus isolation attempts indicated a low level of virus activity. Accordingly, in the 1953 season, ibis adults, eggs, newly hatched young, and young about to leave the nests were collected. The results of the examination of these for neutralizing antibody are presented in table 2. The information obtained from these field collected specimens thus substantiated the earlier hypothesis and was supported by laboratory work which had been conducted over the same period of time.

To demonstrate in the laboratory that transfer to EEE antibody through the egg was possible, an experiment was performed using pigeons which had recovered from laboratory infections with the virus.

Twenty-eight adult pigeons were inoculated subcutaneously with EEE virus. One group of 16 received 21 and the second group of 12 received 68 mouse intracerebral LD_{50}'s. Bloods were collected from the group of 16 birds daily for 5 days and tested for virus. All birds had viremia during the first 2 days. At 66 hours after inoculation, 2 of the 16 had succumbed and the remainder still showed circulating virus. At 90 hours 1 additional bird had died and of the remaining 13 individuals 7 had circulating virus. At 114 hours a 4th bird had died and of the remaining 12 the blood of 2 still contained virus. No bleedings for virus were done after this time. Titrations were performed on the bloods of 4 of these pigeons throughout the period of viremia. The maximum blood virus titers observed varied from $10^{3.3}$ to $10^{5.3}$ LD_{50}'s (table 3).

The bloods of the 12 pigeons receiving 68 LD_{50}'s were tested for virus only at 48 hours after inoculation. All showed the presence of circulating virus.

All the surviving birds were bled 3 weeks after inoculation and their serums tested for neutralizing antibody. The neutralization indexes ranged from 40 to 1480.

Two groups of young were hatched by these birds, 1 group of 4 at 1 to 2 months after infection and another group of 5 at about 5 months. The first group was bled on the 1 day after hatching and again at 1 week. Neutralizing antibodies

TABLE 2.—*Neutralizing antibody in serums of white ibis of varying age and in yolks of eggs collected in 1953.*

Year	Adult	Eggs (yolks)	Newly hatched	4 weeks
1953	20/67*	6/29	4/22	1/20

* 20/67—20 of 67 serums tested showed neutralizing antibodies to EEE virus.

TABLE 3.—*Viremia titers in pigeons following subcutaneous inoculation with 68 LD_{50}'s of EEE virus.*

Pigeon no.	Hours after inoculation: 16	45	66	90	144
9	2.2*	3.3	2.6	1.7	0
11	4.8	4.3	5.3	2.7	Dead
15	4.3	3.3	4.8	Dead	
16	4.3	4.0	3.9	0.6	0
18	3.3	NT†	NT	NT	NT
19	4.5	NT†	NT	NT	NT
20	5.2	NT†	Dead		

* Numbers indicate the log of number of LD_{50}'s per 0.03 ml of blood.
† Not titrated.

TABLE 4.—*Neutralization indexes of young pigeons hatched from eggs laid by immune females.*

	Index dam's serum	Days after hatching: 1–4	5–8	10–14	16–20	24–28	31–33
1	63	20	25	Dead			
2		63			20	Dead	
3		63	Dead				
4	40	20	20	Dead			
5	40		320	50	<5.0	<5.0	<20
6	40		320	160	16	<50	6.0
7	40	50		500	50	13	<20
8	40	160		50		6.3	20
9		160	5.0			2.0	

were present in both bleedings. Since a high mortality resulted from bleeding so soon after hatching, the second group was bled first at 3 to 5 days after hatching and then approximately at weekly intervals. All the nestlings showed the presence of significant amounts of neutralizing antibody at the first bleeding, this antibody persisting until about 3 to 4 weeks of age (table 4).

DISCUSSION

The presence of specific antibody in a host animal has long been used as one of the methods of measuring past activity of a virus. Except in special instances where two or more specimens have been collected from the animal and increases or abrupt decreases in antibody levels have been observed, one should not make assumptions regarding the time at which infection actually occurred.

The observation of antibody in very young birds may lead to the conclusion that virus activity is recent or current, and such a conclusion may lead to an unwarranted search for transmitting vectors associated with the young bird or its immediate environs. Evidence that, at least in certain instances, maternal antibody can be passed from the parent bird through the egg to its offspring emphasizes the importance of interpreting antibody surveys of young bird populations with caution.

The factors imposed on the natural ecology of encephalitis by maternal transfer of antibody would, of course, be difficult to assess. Where a high level of natural endemicity exists, it is possible that young birds to not acquire the infection until after they leave the nest. Thus, they may be of no importance as a host until they reach the young adult stage. On the other hand, because of their availability to arthropods, their susceptibility to infection and the relatively large amounts of virus produced upon infection, nestling birds could be of quite considerable importance in areas where the virus had not been active previously.

SUMMARY

What apparently is maternal antibody has been observed in young nestling birds collected during the course of ecological studies on EEE. Laboratory evidence is presented that maternal neutralizing antibody to EEE can be transferred to the progeny of birds in quantitatively significant amounts. In the young of experimentally infected pigeons this antibody is demonstrable for a period of 3 to 4 weeks after hatching.

CHEMOTHERAPEUTIC STUDIES OF ALLOXAN, DEHYDROASCORBIC ACID, AND RELATED COMPOUNDS IN MURINE HAEMOBARTONELLOSIS

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Murine haemobartonellosis is caused by a rickettsia-like organism, *Haemobartonella muris*, which remains latent in normal rats, but multiplies rapidly in the erythrocytes after removal of the spleen. The mechanism by which the intact spleen prevents the development of the disease remains obscure, but the fact that it does so suggests that it may discharge into the blood stream a chemoprophylactic agent of endogenous origin.

The chemotherapeutic spectrum in this infection is unusual. Certain organic arsenical compounds were shown by Mayer, Borchardt, and Kikuth in 1927 to be highly effective (Kikuth, 1932). This observation has been confirmed repeatedly. A wide variety of antibiotics of fungal origin, as well as PABA, folic acid, atabrine, and several of the newer antimalarial agents are ineffective, but aureomycin and terramycin are highly effective (Stanton, Laskowski, and Pinkerton, 1950).

Recently we have shown that alloxan, in low and presumably nondiabetogenic dosages, is remarkably effective in preventing the development of haemobartonellosis in splenectomized rats (Laskowski, Stanton, and Pinkerton, 1951). In the initial work on which this report was based, rats were maintained on a low sulfur diet. Subsequent work (unreported) has shown that rats on a standard diet of Purina dog chow respond equally well to alloxan, and that daily intraperitoneal injections of 20 mg/kg for 8 days completely prevent the appearance of organisms in the red cell. When the daily dosage is reduced to 10 mg/kg, an occasional rat develops bartonellosis, and in some rats the suppression is only temporary, severe bartonellosis developing about a week after cessation of therapy.

Arsenical compounds are believed to act by combining with SH groups (Voegtlin, Dyer, and Leonard, 1923) and there is evidence that alloxan causes diabetes by inhibiting SH groups (Lazarow, 1946; Conn, Louis, and Johnston, 1949). It therefore appeared that more detailed studies of alloxan and of chemically and pharmacologically related compounds, with particular reference to molecular structure and SH inhibition, might furnish information concerning mechanisms of chemotherapeutic action.

The experiments to be reported here were designed to answer the following questions: (1) What components of the alloxan molecule are essential for its therapeutic action? (2) Which of the various agents with proven or strongly suspected ability to inhibit SH groups are capable of suppressing haemobartonellosis? (3) Can the therapeutic action of alloxan be reversed by those agents which reverse its diabetogenic action? (4) Do alloxan and aureomycin owe their therapeutic activities to the same mechanism? (5) Is there a relation between alloxan bartonellostasis and

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the suppressive action of the spleen on *H. muris*? (6) Does alloxan have chemotherapeutic activity in infections other than murine bartonellosis?

MATERIAL AMD METHODS

Latently infected (carrier) rats of the St. Louis University strain were used. Previous work with many rats of this strain has shown that splenectomy is invariably followed by severe haemobartonellosis. Organisms appear in or on the erythrocytes by the 2nd or 3rd day after the removal of the spleen, reach the maximum number on the 5th or 6th day, and disappear in surviving animals by the 9th or 10th day. The highest daily bartonella counts, made at the apex of the disease, are rarely below 100 organisms per 100 erythrocytes, and the mortality is about 50%. The effect of various compounds on the development of the disease was determined by comparing the number of organisms in Giemsa-stained blood films from treated and untreated animals. These observations were made daily for about 12 days, starting on the day following splenectomy. The bartonella counts were based on the examination of 400 erythrocytes and were recorded as organisms per 100 erythrocytes.

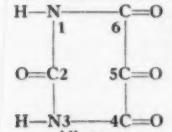
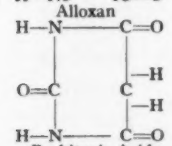
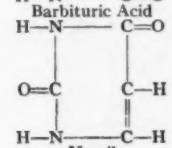
All drugs, unless otherwise stated, were dissolved in appropriate amounts of distilled water and given by the intraperitoneal route. Details concerning dietary and dosage factors are given in the tables or text accompanying each group of experiments. Because of the uniform incidence and course of haemobartonellosis in splenectomized animals, and the "all or none" nature of most of the observed effects, relatively small numbers of animals often sufficed to give significant results.

RESULTS

Compounds chemically related to alloxan

Barbituric acid and uracil.—Structurally, barbituric acid differs from alloxan in the substitution of H_2 for O at position 5. In uracil, the oxygen atoms at positions 4 and 5 are replaced by hydrogen atoms (see table 1). These compounds were tested for bartonellostatic action, and also for possible inhibition of the activity of alloxan. Twenty-one carrier rats were splenectomized, divided into appropriate groups, and treated with alloxan, barbituric acid, uracil, barbituric acid plus alloxan and uracil plus alloxan. All drugs were started about 24 hours before splenectomy, and daily injections at the dosage levels indicated were given for 9 days. All animals in this experiment were maintained on a previously described

TABLE 1.—*Effect of alloxan and related compounds on haemobartonellosis in splenectomized rats.*

Structural formula	Rat no.	Drug	Dosage	Highest bartonella count (per 100 rbc)	Outcome
 Alloxan	1	Alloxan	20 mg/kg daily	0	S
	2			0	S
	3			0	S
	4	Barbituric acid	40 mg/kg daily	170	S
	5			246	D
	6			296	S
 Barbituric Acid	7	Barbituric acid and alloxan	40 mg/kg 1–3 min. before alloxan (20 mg/kg)	0	S
	8			0	S
	9			0	S
	10	Uracil	120 mg/kg daily	259	D
	11			201	S
	12			283	S
	13			133	S
 Uracil	14	Uracil and alloxan	60 mg/kg 1–3 min. before alloxan (20 mg/kg)	0	S
	15			0	S
	16	None	Haemobartonella control	244	S
	17			231	S
	18			252	S
	19			205	D
	20			262	S
	21			168	D

S, survived.
D, died of haemobartonellosis.

low sulfur diet from the day of splenectomy until the termination of the observation period 21 days later. The results of this experiment (table 1) suggest that 3 adjacent C=O groups are essential for bartonellostatic activity of the type shown by alloxan.

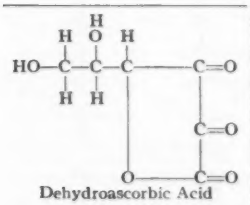
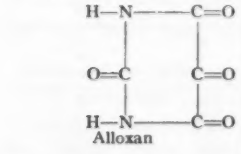
Dehydroascorbic acid.—This compound resembles alloxan in having 3 adjacent C=O groups (see table 2). Large initial doses, as shown by Patterson (1948) and confirmed by us, cause a severe reaction characterized by hyperactivity, flushing of the skin and discharge of fluid from the nose, followed in a few minutes by collapse and often by death. For this reason it was necessary to give an initial dose of 200 mg/kg. Following this "desensitizing" dose, daily injections of 700 mg/kg were fairly well tolerated and resulted in practically complete suppression of the multiplication of *H. muris*.

Table 2 summarizes the results of one of three separate experiments in which this effect was noted. In the experiment represented by this table, the desensitizing dose was given about 24 hours before splenectomy, and daily injections of 700 mg/kg were started about 48 hours after splenectomy and continued for 7 days. In a second experiment the desensitizing dose was given 1 to 2 hours after splenectomy, and 7 daily injections of 700 mg/kg were given, starting 24 hours after splenectomy. Almost complete inhibition of the multiplication of the organism was again noted, and only 1 of the group of 10 animals died (on the 12th day after splenectomy). A repetition of the second experiment gave identical results, except that 2 of the 10 treated animals died, apparently from drug toxicity, on the 11th and 12th days after splenectomy.

When doses of 500 mg/kg were given, only partial suppression of the bartonellemia was found. It should be noted that while dehydroascorbic was practically as effective as alloxan, the dosages required were somewhat toxic (6 deaths among 30 treated animals) and that the effective dosage levels were probably high enough to cause pancreatic injury.

All rats used for these experiments involving dehydroascorbic acid were maintained on Purina dog chow.

TABLE 2.—*Effect of dehydroascorbic acid on haemobartonellosis in splenectomized rats.*

Structural formula	Rat no.	Drug	Daily dosage	Highest bartonella count (per 100 rbc)	Outcome
 Dehydroascorbic Acid	1	Dehydroascorbic acid	200 mg/kg on 1st day, 700 mg/kg thereafter	0	S
	2			0	S
	3			0	D—8th day
	4			0	D—8th day
	5			1	S
	6			1	D—6th day
	7			2	S
	8			0	D—4th day
	9			0	S
	10			4	S
 Alloxan	11	None	Haemobartonella control	158	S
	12			1	D*—1st day
	13			199	S
	14			358	D†
	15			190	S
	16			339	S
	17			342	D†
	18			201	S
	19			34	D†

S, survived.
D, died of drug toxicity.
D*, cause of death undetermined.
D†, died of haemobartonellosis.

SH inhibitors

Many enzymes contain active sulfhydryl groups, and the inhibition of these groups is currently believed to be of importance in blocking a number of metabolic pathways. The diabetogenic action of alloxan has been explained on the basis of interference with SH enzymes in the Beta cells of the pancreas (Lazarow, 1946; Conn, Louis, and Johnston, 1949). Sulfhydryl inhibitors apparently vary in their selectivity and mode of action, and for this reason several representative types were tested for possible bartonellostatic activity.

Bromobenzene and naphthalene.—The detoxification of these compounds in the body, by the formation of mercapturic acids, results in a lowering of the thiol group reservoirs (West, 1940; Stekol, 1939).

After determining approximately the maximal dose of these agents which would permit survival, 15 rats were placed on the low sulfur diet previously described. To the diet of 5 of these rats 1% bromobenzene was added. Another group of 5 rats received the low sulfur diet supplemented by 1% naphthalene, the remaining 5 rats serving as controls. After 5 days, all 15 animals were splenectomized, and daily bartonella counts were made for 13 days. There was a 24-hour delay in the appearance of organisms in the red cells of treated animals of both groups.

From the average daily bartonella counts in each group, it was apparent that in both treated groups the bartonellemia developed more slowly, and the parasite counts reached average peak levels less than half as high as that noted in the controls. Organisms persisted in the blood of both treated groups, however, for 4 days after they had disappeared from the blood of the controls. It seems probable then that the effect of both bromobenzene and naphthalene was to make the parasitemia less severe but more prolonged. Because of the high toxicity of the compounds, however, no definite conclusions could be drawn.

Glutathione given intraperitoneally in daily doses of 660 mg/kg for 8 days did not cause the appearance of bartonellas in the circulating blood of 8 rats with intact spleens.

P-chloromercuribenzoic acid and furacin.—These agents may be considered as specific thiol inhibiting agents. The former is believed to differ from the trivalent organic arsenicals in that it inactivates SH groups too far apart to form the S—S linkage (Barron, 1951). Furacin (5 nitro-2-furaldehyde semicarbazone) has been found to selectively inhibit certain bacterial enzymes containing SH groups, including urease and malic, succinic, lactic and 2-glycero phosphatase dehydrogenases (Green, Heath, and Yall, 1951).

In testing these agents, rats were started on the low sulfur diet 24 hours before splenectomy, and drug injections were begun 24 hours after splenectomy. The results of representative experiments are seen in table 3. *P*-chloromercuribenzoic acid effectively prevented the multiplication of *H. muris* during the first few days after splenectomy, but only when given in toxic and usually fatal doses. Nontoxic doses of this agent were ineffective. The complete absence of bartonellas from the blood of the animals receiving large doses is significant, even though they died on the 4th, 5th, or 6th days after splenectomy, since the control animals had high parasite counts on these days. Furacin at the dosage levels tested was completely ineffective.

X radiation.—There is evidence that X radiation, by the formation of hydrogen peroxide from water, inactivates SH enzymes (Barron, Dickman, Muntz,

TABLE 3.—*Effect of furacin and p-chloromercuribenzoic acid on haemobartonellosis in splenectomized rats.*

Rat no.	Drug	Dosage	Highest bartonella count (per 100 rbc)	Outcome
1	Furacin	50 mg/kg daily	190	S
2			339	S
3			342	D
4			201	S
5			34	D
6	P-chloromercuribenzoic acid	50 mg/kg daily	0	D*—5th day
7			0	D*—6th day
8			0	S
9			0	D*—4th day
10			0	D*—5th day
11	None	Haemobartonella control	158	S
12			199	S
13			358	D
1	P-chloromercuribenzoic acid	25 mg/kg daily	0	D*—5th day
2			0	D*—5th day
3			1	D*—5th day
4		12.5 mg/kg daily	57	D*—6th day
5			1	D*—2nd day
6			0	D*—4th day
7		6.25 mg/kg daily	256	S
8			159	S
9			162	S
10	None	Haemobartonella control	201	S
11			163	S
12			191	D
13			187	D
14			196	S

S, survived.
D, died of haemobartonellosis.
D*, death due to toxicity of drug.

and Singer, 1949). At dosage levels of 350 r or 750 r, given either on the day preceding or the day following splenectomy, X radiation showed no significant effect on the multiplication of *H. muris* in the erythrocytes. (In these experiments 10 rats were used in each group, together with 20 control animals. The lower dosage was nonlethal, while the higher dosage resulted in about 50% mortality from radiation injury, most deaths occurring between the 5th and 9th days after radiation.) Similar doses of radiation did not cause bartonellas to appear in significant numbers in the blood stream of nonsplenectomized rats. The bartonellostatic effect of alloxan was not significantly enhanced or neutralized by radiation at these dosage levels. In another experiment, 12 rats were given a total of 2000 r each over a period of 3 months, in divided doses of 350 r. The 5 rats surviving this injury were splenectomized, and typical bartonellosis developed in each animal. All rats used for radiation studies were maintained on a diet of Purina dog chow.

Cortisone and ACTH.—Cortisone was given in daily doses of 7.5 mg/kg. One group of 10 rats received this dose for 7 days before splenectomy and for 5 days after splenectomy. These animals showed no evidence of bartonellosis previous to splenectomy, but all developed typical bartonellosis following splenectomy. Another group of 10 rats received similar daily doses of cortisone, starting on the day after splenectomy; again the course of the disease was not altered. Cortisone-treated rats were maintained on Purina dog chow.

Similarly, ACTH, at daily dosage levels of 3 mg/kg started 3 days before splenectomy and continued for 11 days, had no demonstrable effect on the course of haemobartonellosis, as judged by the parasite counts, in 8 rats.

Effect of "reversal" drugs

The diabetogenic action of alloxan may be prevented by previously injecting BAL, cysteine HCl, glutathione, or boric acid (Lazarow, 1947; Lazarow, 1946; Rose and Gyorgy, 1949). From the data presented in tables 4 and 5, it is clear that the bartonellostatic action of alloxan was neutralized by BAL and by boric acid, but not by the other agents. Boric acid is believed to form a complex molecule with alloxan which renders it chemically inactive (Rose and Gyorgy, 1949). The neutralizing action of BAL probably depends on its ability to combine, through its SH groups, with alloxan, or perhaps even to withdraw alloxan from combination with essential SH containing enzymes.

The partial reversal of alloxan activity by cysteine HCl buffered with sodium borate (table 4) was apparently the result of the borate component, since this effect was not noted when NaOH was used as a neutralizing agent (table 5).

Effect of alloxan on other diseases

Alloxan in daily dosages of 100 mg/kg was given to a group of 12 mice infected with *Trypanosoma rhodesiense*. The result was a 24-hour prolongation of the incubation period, and a reduction of the average daily parasite count in the peripheral blood to about half that noted in the 12 control mice. In similar doses, alloxan did not modify the course of fixed rabies virus infection in mice, St. Louis encephalitis in mice, *Rickettsia mooseri* infection in fertile eggs, or sarcoma 37 in mice. In mice infected with *Pasteurella tularensis*, alloxan in similar doses apparently caused more rapid death, most of the treated animals dying on the 2nd day, while the majority of the untreated animals died on the 3rd day.

DISCUSSION

It is not possible as yet to discuss the results of these experiments in terms of the exact mechanisms involved. The therapeutic effectiveness of arsenicals

TABLE 4.—*Effect of reversal drugs on the therapeutic activity of alloxan and aureomycin.*

Reversal drug 1–3 min. before alloxan or aureomycin	Aureomycin 4.17 mg/kg twice daily	Highest bartonella count (per 100 rbc)	Outcome	Alloxan 20 mg/kg once daily	Highest bartonella count (per 100 rbc)	Outcome
None (Bartonella control)	None	401	D	None	179	S
		216	D		227	D
		208	S		278	S
		272	S		183	S
None (drug control)	Yes	0	S	Yes	0	D*
		0	S		0	S
		0	S		0	S
		0	S		0	S
Bal 0.1 cc 10% soln. in peanut oil per 300 g rat	Yes	<1	S	Yes	177	S
		<1	S		251	S
		<1	S		183	S
		<1	S		12	D*
Cysteine-HCl 91.2 mg/kg (sod. borate buffer pH4)	Yes	0	S	Yes	1	S
		0	S		29	D*
		0	S		2	D*
		0	S		2	S
Glutathione 250 mg/kg (pH 4 with NaOH)	Yes	0	S	Yes	0	S
		0	S		0	S
		0	S		0	S
		0	S		0	S
Insulin 0.1 unit per 300 g rat	Yes	0	S	Yes	0	S
		0	S		0	S
		0	S		0	S
		0	S		0	S

S, survived.
D, died of haemobartonellosis.
D*, cause of death undermined.

TABLE 5.—*Effect of reversal drugs on alloxan and aureomycin therapeutic activity.*

Reversal drug 1–3 min. before alloxan or aureomycin	Alloxan 20 mg/kg once daily	Highest bartonella count (per 100 rbc)	Outcome	Aureomycin 4.17 mg/kg twice daily	Highest bartonella count (per 100 rbc)	Outcome
None (bartonella control)	None	201	S	None	222	S
		163	S		373	S
		191	D		167	S
		187	D		234	D
		196	S		184	D
None (drug control)	Yes	0	S	Yes	0	S
		0	S		0	S
		0	S		<1	S
		0	S		0	S
					<1	S
Boric acid 30 mg/kg	Yes	71	S			
		0	S			
		2	S			
		201	S			
Boric acid 60 mg/kg	Yes	13	S	Yes	<1	S
		9	S		0	S
		86	S		<1	S
		103	S		0	S
		72	S		<1	S
Boric acid 90 mg/kg	Yes	0	S			
		191	S			
		0	S			
		263	S			
		289	S			
Cystine-HCl (Merck) 100 mg/kg (pH 4.0 with NaOH)	Yes	0	S			
		0	S			
		0	S			
		0	S			
		0	D* 4th day			
		0	S			
		0	S			
		0	D* 5th day			
		0	D* 5th day			

S, survived.
D, died of haemobartonellosis.
D*, death due to toxicity of drugs.

and the stimulating effect of gluthathione combined with ascorbic acid on the multiplication of *Bartonella bacilliformis* in vitro (Geiman, 1941) suggest the importance of SH groups in the multiplication of organisms of this group. This concept is further supported by the effectiveness of such thiol group inhibitors as alloxan, dehydroascorbic acid and *p*-chloromercuribenzoic acid, and by the partial effectiveness of general thiol depletion by bromobenzene and naphthalene. Assuming that SH group inhibition is important in the bartonellostatic action exhibited by these compounds, it is not clear whether the effect is exerted on the enzymes of the parasite or on those of the host cell. Since some powerful thiol inhibitors are devoid of bartonellostatic activity, it is probable that specific enzymes are involved.

Alloxan shows bartonellostatic action in doses much lower than those necessary for diabetogenesis. Since its diabetogenic action is reversed by cysteine or glutathione, while its bartonellostatic action is not, it would appear that the two activities are mechanistically not completely identical. It should be pointed out, however, that in our experiments all drugs were given intraperitoneally, while in the experiments showing reversal of alloxan diabetogenesis by cysteine and gluthathione, the intravenous route was used.

It is apparent that the bartonellostatic action of aureomycin differs from that of alloxan and dehydroascorbic acid, since the former was not significantly reversed by BAL. This is in harmony with the observation that in experimental spirochetal infections, BAL reversed the therapeutic action of

penicillin, bacitracin and chloramphenicol, while it had no effect on the action of streptomycin, terramycin, or aureomycin (Ercoli, Gosford, Carminati, Kley, and Schwarz, 1951). The inability of monothiols (cysteine and glutathione) to reverse the therapeutic action of alloxan likewise finds its parallel in the inability of these agents to reverse the action of penicillin, bacitracin and chloromphenicol (Ercoli et al., 1951).

We are as yet unable to relate the therapeutic action of thiol inhibitors such as alloxan and dehydroascorbic acid to the normal activity of the spleen. It is of interest to note, however, that Ungar (1947) has described the isolation from the spleen of two agents (splenin A and splenin B), the former apparently inhibiting the autooxidation of ascorbic, acid, while the latter accelerates its oxidation.

It seems possible that the equilibrium between reduced and oxidized ascorbic acid, which in turn depends on glutathione and on the general thiol group reservoirs, may influence the multiplication of *H. muris*, but no experimental data on this point are available.

SUMMARY AND CONCLUSIONS

The chemotherapeutic activity of alloxan in murine bartonellosis appears to depend on the presence of three adjacent C=0 groups, since barbituric acid and uracil are ineffective. Dehydroascorbic acid, which also contains three adjacent C=0 groups is equally effective, but only when given in large and definitely toxic dosages. *P*-chloromercuribenzoic acid is also effective at toxic dosage levels.

Bromobenzene and naphthalene are partially effective. Furacin, X radiation, cortisone, and ACTH are ineffective. The therapeutic acition of alloxan is reversed by BAL, but not by monothiols (cysteine and glutathione). The therapeutic action of aureomycin is not affected by BAL.

Thus it appears that some, but not all, thiol group inhibitors have bartonellostatic action, but that none is as effective as alloxan and the organic arsenicals. The relation of the observations made to the bartonellostatic action of the intact spleen is not clear.

Alloxan was partially effective in *Trypanosoma rhodesiense* infection, but ineffective in fixed rabies, St. Louis encephalitis, murine typhus, tularemia and sarcoma 37.

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EXPERIMENTAL NEPHRITIS DUE TO TYPE SPECIFIC STREPTOCOCCI

I. THE EFFECT OF A SINGLE EXPOSURE TO TYPE 12 STREPTOCOCCI

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Acute glomerulonephritis is now considered to be a nonsuppurative complication of infections with hemolytic streptococci of Lancefield's group A, although the exact mechanism by which the disease develops has not been clearly defined. As early as 1917, Ophuls[1] indicated that only certain strains of streptococci appeared to have a definite nephritogenic action in man. Rammelkamp, Weaver and Dingle[2] have reported evidence showing that the incidence of acute glomerulonephritis following group A streptococcal infections varied considerably from year to year, while that of acute rheumatic fever remained fairly constant. They postulated that this difference was due to the random appearance of specific types of streptococci which were nephritogenic. Recent epidemiological studies of widely separated outbreaks of nephritis have amply supported this viewpoint.

Kempe, Ohmstead and Curnen[3] isolated four strains of type 12 streptococci from an outbreak of acute nephritis in a boys' school in Connecticut in 1951. In addition to 4 cases of nephritis among the 96 boys, there were 21 pupils with microscopic hematuria. No other typeable group A streptococci were isolated. Manser and Wilson[4] have recently described an epidemic of acute streptococcal pharyngitis from Australia, during which 8 cases of classical acute nephritis and 30 to 40 patients with edema and albuminuria were observed in a town of 8000 population. Thirty-five typeable strains of group A streptococci were isolated from throat cultures. Of these, 16 were type 12, 10 were type 22 and 9 belonged to other types. Rantz[5] isolated 14 type 12 strains from middle ear exudates obtained from 84 patients with otitis media in the San Francisco area from 1939 to 1943 Two of these 14 individuals subsequently developed acute glomerulonephritis. Wertheim et al[6] have reviewed case records of nephritis patients in 2 New York hospitals from 1936 to 1942. Thirty-nine strains of group A strepto-

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cocci were isolated, of which 20 were identified as type 12.

Rammelkamp and Weaver[7] have recently reported the results of a 4-year study of sporadic outbreaks of nephritis in the United States. They found that in a group of 31 patients observed in widely separated areas, type 12 streptococci were associated with 26 cases (84%), type 25 was related to 4 cases and type 4 to one case. In addition, type 4 streptococci were isolated from 2 patients with nephritis during an outbreak in Hawaii. The same authors[7] observed 2366 young adults for 3 weeks following the onset of acute pharyngitis due to group A streptococci. Only one patient, infected with type 12 streptoccoci, developed nephritis. The authors indicated that type 12 strains may vary in their nephritogenic capacity. Rammelkamp[8] cites the occurrence of 37 cases of acute nephritis in a nursery school where a high type 12 carrier rate had developed. During the spring of 1952, Rammelkamp et al[9] studied a large epidemic of nephritis in a military population in which the infecting strain was type 12. Reed[10] has studied an epidemic of acute nephritis in rural Nova Scotia in which 22 cases were observed during a 4-month period. Seventeen cases cocurred in those under age 17 in a village of 360 people. School and family illness records from November 1951 to March 1953 showed a very high incidence of sore throat. Throat cultures from 153 persons yielded 56 group A streptococci, of which 53 were type 12 strains. The type 12 carrier rate among those under 17 was 4 times that of the older age group. These rates showed some correlation with the incidence of nephritis and sore throat during the winter.

The evidence presented above indicates that an attack of acute glomerulonephritis is much more likely to follow infections with type 12 streptococci than with other serological types. This evidence and the availability of highly nephritogenic type 12 strains from the Nova Scotia epidemic stimulated the studies to be reported. Our primary purpose was to determine if localized infection of rabbits with type 12 streptococci would induce a clinical picture analagous to that of acute glomerulonephritis in man.

MATERIALS AND METHODS

Animals.—New Zealand white rabbits 4 to 5 lb. in weight were used in all experiments. Diet was restricted to Purina rabbit chow, a complete ration. Water was provided freely at all times.

Cultures.—Type 12 streptococci used in these experiments were obtained from the following sources: strains H8, H3, 8 and 35 from the Nova Scotia epidemic;[10] strain 7–56 from a family outbreak of nephritis in Cleveland (case 7 of Rammelkamp);[7] strain 7–54 from an outbreak in Bainbridge, Maryland (case 27 of Rammelkamp).[7] Types 1, 3 and 10 were obtained from the Laboratory of Hygiene, Ottawa and types 17 and 25 were isolated during the Nova Scotia epidemic. Cultures of *Streptococcus viridans*, *Micrococcus pyogenes* var. *aureus* and *Escherichia coli* were freshly isolated from clinical specimens. All organisms were incubated for 24 hours at 37 C in Todd-Hewitt broth[11] before use. All bacteria were sensitive in vitro to the antibiotic used to terminate experimental infections.

Infection.—The majority of cases of acute glomerulonephritis in man are preceded by a streptococcal infection of short duration localized in the pharynx. This localized infection was simulated in rabbits by subcutaneous injection

7. Rammelkamp, C. H. and Weaver, R. S. 1953, Acute glomerulo-nephritis. The significance of the variations in the incidence of the disease. J. Clin. Invest. **32**: 345–358.
8. Rammelkamp, C. H. Personal communication.
9. Rammelkamp, C. H., Stetson, C. A., Krause, R. M., Kohen, R. and Perry, W. D. To be published.
10. Reed, R. W. 1953, An epidemic of acute nephritis, Canad. M.A.J. **68**: 448–455.
11. Todd, E. W. and Hewitt, L. J. 1932, A new culture medium for the production of antigenic streptococcal hemolysin. J. Path. & Bact. **35**: 973–974.

of 0.1 ml of whole culture in the shaved dorsal lumbar region. Infection was terminated after 10 days by intramuscular injection of penicillin.

Filtrates.—Filtrates of the same cultures were injected intravenously to determine if any nephritogenic substance produced was extracellular. Todd-Hewitt broth cultures were centrifuged and the supernatant passed through Seitz "EK" filter discs. Sterility tests and streptolysin "O" titrations were done on all batches of filtrate before use. All batches showed less than 1 unit per ml of streptolysin O.

Antistreptolysin O.—ASLO titrations on serums from selected groups of rabbits were done at regular intervals. Standard streptolysin O reagent (Difco) was used in these titrations, which were done by their recommended procedure.

Urinalysis.—Urines were collected for 24-hour periods and abnormal constituents observed microscopically were calculated on this basis. Albumin was determined by Robert's method.[12] Since some normal animals showed albumin in urine diluted $\frac{1}{4}$, all tests were done on dilutions of $\frac{1}{5}$ or more. Results were recorded as follows: Albumin in $\frac{1}{5}$ dilution = +, in $\frac{1}{6}$ = 2+, in $\frac{1}{7}$ = 3+, in $\frac{1}{8}$ = 4+.

Peripheral arterial pressure.—Blood pressures were determined using an ear capsule method modified slightly from that of Reed and Kropp.[13] These authors showed that carotid cannula and ear capsule readings taken simultaneously gave identical fluctuations in normal and in adrenalin-treated rabbits. Readings of lower magnitude were obtained by the ear capsule method, with average normal peripheral blood pressures of 20 to 30 mm Hg. We have found the same range in normal animals. Since blood pressure readings formed a very important part of the experimental procedure in this investigation, the apparatus and methods will be described in detail.

A diagram of the apparatus is shown in figure 1. A 3-inch length of $\frac{3}{4}$ inch brass tubing (A) was sealed at the upper end with a lucite window and at the lower end with a piece of condom rubber, laid flat over the orifice and held in place by several layers of liquid latex around the tube. This capsule was connected by a side-arm to a mercury manometer (G) and by a T-tube to a sphygmomanometer bulb (F), thus forming an airtight system. The brass tube or capsule was mounted in an adjustable screw clamp supported on an iron stand. A lucite plate (D) was supported on an iron ring with a stereoscopic microscope (C) mounted over it. Light from a microscope lamp was directed up through the lucite plate and the capsule to the microscope by a mirror

The rabbit was held in a plaster mold with the ears protruding through a hole in the top, as suggested by Reed & Kropp.[13] Animals soon learned to remain quiet in the holder. Those whose respiratory rate increased or which became restless were not read until later. Occasionally it was necessary to dilate constricted arteries by gently rubbing the ears. The necessity for vasodilation of constricted arteries during blood pressure measurements was emphasized by Grant and Rothschild.[14]

The ear was placed on the lucite plate and the capsule lowered until it barely touched the ear over the bifurcation of the central artery, about 5 cm from the tip of the ear. The bulb (F) was compressed until the artery was completely occluded, then air admitted slowly. At the exact moment when blood reappeared in the artery, a reading was taken on the manometer. Readings were facilitated by having the 30 mm mark at the level of the microscope eyepieces, and by mounting a large magnifying lens (E) at that level. As reported by Grant and Rothschild,[14] when the central artery was occluded for a short time and then released, the ear became temporarily flushed due to reactive hyperemia. Therefore, a one minute interval was allowed between readings so this reaction could subside. Ten consecutive readings were taken and the average recorded. Fluctuation between these consecutive readings was never more than 4 mm Hg.

EXPERIMENTAL RESULTS

Type 12 streptococci

Sixteen rabbits were observed for 5 days to establish normal blood pressure readings and urinalysis, then divided at random into groups of 4. The animals in group 1 received intravenous injections of filtrate prepared from strain H8 cultures. Group 2 animals were injected intravenously with sterile Todd-Hewitt broth. Animals of group 3 were infected

12. Todd, J. C., Sanford, A. H. & Wells, B. B. 1953, Clinical Diagnosis by Laboratory Methods, ed. 12, W. B. Saunders Company, p. 77.

13. Reed, G. B. and Kropp, B. 1944, A simple apparatus for determining peripheral arterial pressure in the rabbit's ear. J. Lab. & Clin. Med. **29**: 214–217.

14. Grant, R. T. and Rothschild, P. 1934, A device for estimating blood pressure in a rabbit. J. Physiol. **81**: 265–269.

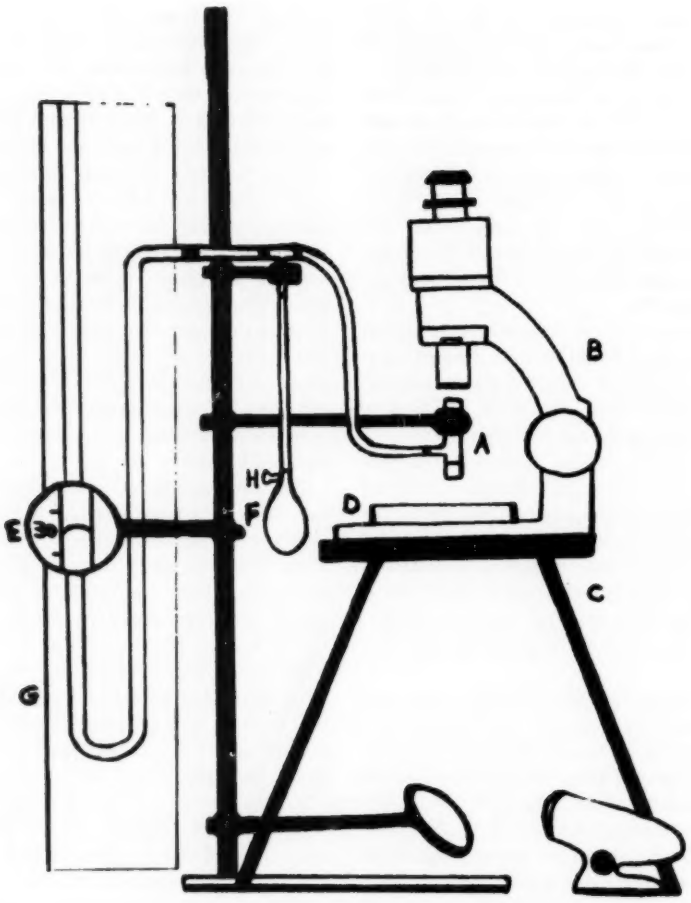

FIG. 1.—Apparatus used for determining peripheral arterial blood pressure of rabbits.

subcutaneously with 0.1 ml of whole culture of strain H8, and later treated with intramuscular penicillin. Group 4 were untreated normal control animals. The treatment schedule for all groups is shown in table 1.

Blood pressure determinations were done every 2nd or 3rd day on all animals, with results as shown in figures 2 to 5. Normal blood pressures on all animals were between 25 and 30 mm Hg. After the 3rd injection of filtrate, blood

TABLE 1.—*Treatment schedule of groups 1 to 4. Organism:* Str. pyogenes *type 12 (strain H8).*

Day	Group 1 (filtrate)	Group 2 (sterile broth)	Group 3 (infection)	Group 4 (normal)
1–5	Control period for all four groups			
6	1 ml filt. IV	1 ml broth IV	0.1 ml cult. subQ	Untreated throughout
9	1 ml filt. IV	1 ml broth IV		
12	1 ml filt. IV	1 ml broth IV		
15	2 ml filt. IV	2 ml broth IV		
16			80,000 U penicillin	
18	2 ml filt. IV	2 ml broth IV	80,000 U penicillin	
20			80,000 U penicillin	
21	3 ml filt. IV	3 ml broth IV		

IV = intravenously.
subQ = subcutaneously.

TABLE 2.—*Urinalyses of group 1 rabbits. Treatment:* Str. pyogenes (*H8*) *filtrate intravenously.*

Days after treatment	Rabbit #23 Urine, 24 hr		Rabbit #58 Urine, 24 hr		Rabbit #55 Urine, 24 hr		Rabbit #18 Urine, 24 hr	
	Alb.	RBC	Alb.	RBC	Alb.	RBC	Alb.	RBC
1–16*	0	—	0	—	0	—	0	—
18	+	—	+	—			+	—
19					+	—		
24	+	—	+	—			+	—
25					+	—		
26	+	—	+	—			+	—
28			0	—	+	—		
29	+++	660,000					+	—
31	++	352,000	+	93,000	++	5,000		
33	++	547,000	0	42,000	++	20,000	+	—
35	++	174,000	0	25,000			+	—

Alb. =albumin.
RBC =red blood cells.
* Urines from each rabbit were examined six times during this period and were normal.

pressures of group 1 animals showed a rapid increase, particularly in rabbit #58 (fig. 2). This animal's blood pressure rose to 40 mm Hg 10 days after the 1st injection and to 45 mm 10 days later, maintaining this level until the animal was sacrificed on the 40th day. The other three rabbits showed more gradual increases, which reached a lower peak but which were maintained for 30 days. No increase in blood pressure above 30 mm was noted in rabbits of group 2 (fig. 3) receiving sterile broth intravenously. No change in blood pressure of group 3 rabbits (fig. 4) was observed until 17 days after infection, when 3 of the 4 showed sharp increases, especially rabbit #20. These increased blood pressures were maintained until the animals were sacrificed 35 days after infection. Rabbit #24 in this group did not develop hypertension. Blood pressures of the untreated control group (fig. 5) remained under 30 mm Hg for the entire period of observation.

The urines of all animals were examined at 2- to 5-day intervals for 35 days after the beginning of treatment. The findings in group 1 (filtrate) rabbits are presented in table 2. All animals showed 1+ albumin 18 to 19 days after the first injection of filtrate, i.e., 1 to 10 days following the onset of hypertension. This increased to 2+ in rabbits #23 and #55 at 20 and 12 days after the onset of hypertension which now had reached its peak. One rabbit (#23) began to excrete a moderate number of red cells after 20 days of hypertension, while #55 excreted very few. Microscopic hematuria

TABLE 3.—*Urinalyses of group 3 rabbits. Treatment: Local infection with* Str. pyogenes (*H8*).

Days after infection	Rabbit #72 Urine, 24 hr		Rabbit #65 Urine, 24 hr		Rabbit #20 Urine, 24 hr		Rabbit #24 Urine, 24 hr	
	Alb.	RBC	Alb.	RBC	Alb.	RBC	Alb.	RBC
1–14*	0	—	0	—	0	—	0	—
15	+	—	+	—	0	—	0	—
19							0	10,000
20	+	—	+	—	+	—		
22	+	—	+	—	+	—	0	136,000
27					+	1,020,000	0	40,000
29	+	282,000	0	—			0	34,000
31					++	634,000		
32	0	—	0	—	0	104,000	0	—
33			0	—	++	180,000		
35	+	208,000			++	290,000	0	—

Alb. =albumin.
RBC =red blood cells.
* Urines from each rabbit were examined five times during this period and were normal.

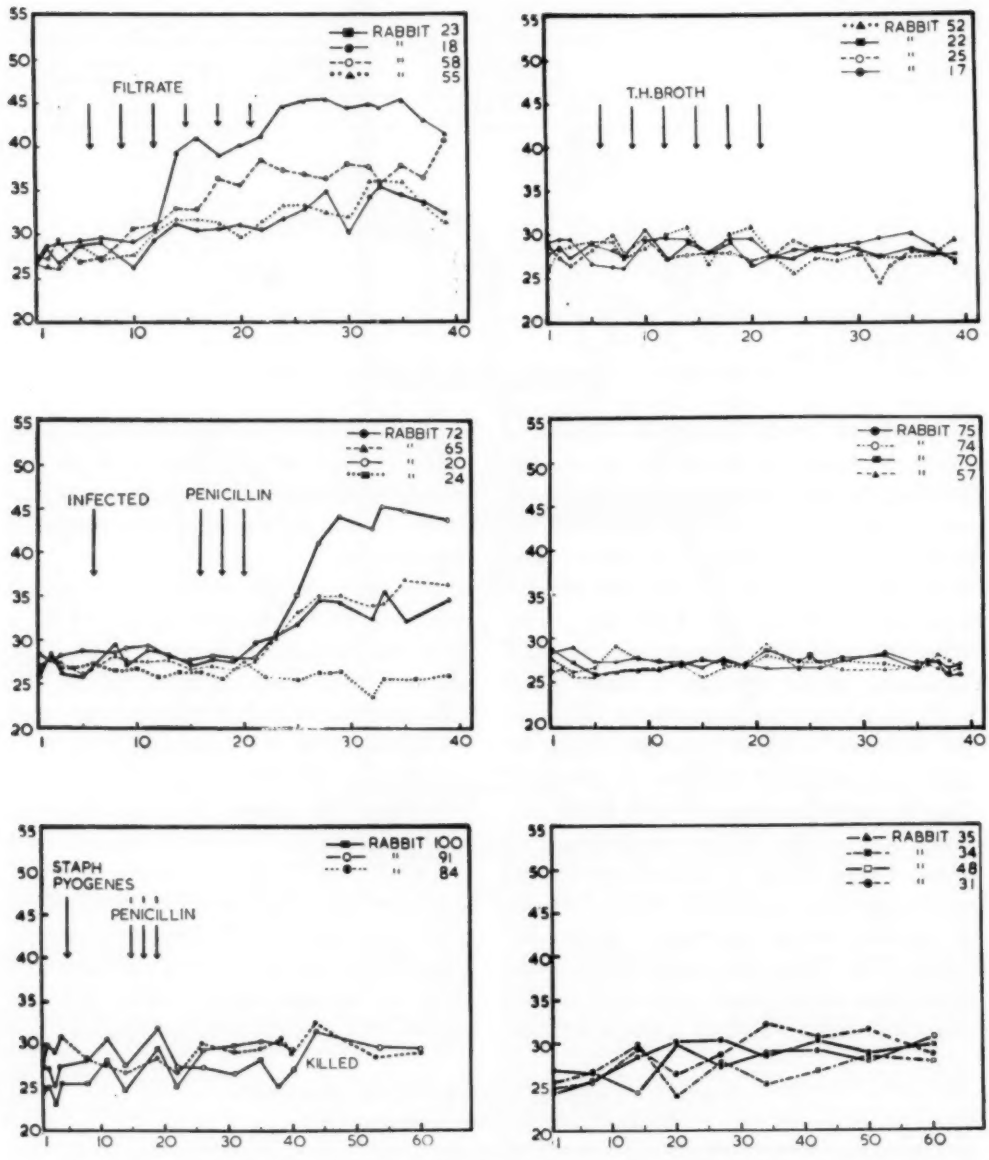

FIG. 2–7.—Peripheral arterial blood pressure in mm Hg (ordinate) plotted against time in days (abscissa) of groups of rabbits treated as follows:

FIG. 2.—(Upper left) Rabbits of group 1. Intravenous filtrate of type 12 cultures.

FIG. 3.—(Upper right) Rabbits of group 2. Intravenous sterile Todd-Hewitt broth.

FIG. 4.—(Center left) Rabbits of group 3. Subcutaneous infection with type 12 culture.

FIG. 5.—(Center right) Rabbits of group 4. No treatment.

FIG. 6.—(Lower left) Rabbits infected subcutaneously with *M. pyogenes*.

FIG. 7.—(Lower right) No treatment.

TABLE 4.—*Antistreptolysin O titers of rabbits locally infected with* Str. pygogenes (*H8*).

Rabbit no.	Days after infection							
	4	8	12	16	20	25	30	35
77	<12	<12	<12	625–833	50–100	12–50	—	—
99	<12	<12	<12	333–500	50–100	12–50	12–50	12–50
19	<12	12–50	166–250	333–500	12–50	12–50	12–50	<12

was detected in 3 of the 4 until the 35th day. Table 3 shows the urine findings of group 3 (infected) animals. Albuminuria appeared in 3 of 4 rabbits at the same time as hypertension but increased to 2+ only in #20, which showed the highest blood pressure, and also the highest and most constant excretion of red cells. Two rabbits (#72 and #65) showed only sporadic 1+ albuminuria with slight hematuria in one case and none in the other. Rabbit #24 did not develop hypertension or albuminuria but did excrete some red cells. The urines of group 2 (broth) and group 4 (untreated) animals were examined 10 times in 40 days and were normal at all times.

All animals were sacrificed 35 days after treatment began and postmortems were done. The kidneys of all group 1 (filtrate) animals were soft with numerous hemorrhages from pinpoint size to 5 mm diameter. These were especially marked in rabbit #58. There was noticeable fluid in the peritoneal cavity in 3 of these 4 animals. Microscopically, the kidneys showed evidence of cellular degeneration and regeneration confined mainly to the first convoluted tubule. Glomerular capillaries were dilated and congested, with an abnormal number of round cells in Bowman's capsule. No other glomerular abnormality was observed. A diagnosis of healing lower nephron nephrosis was made. Gross and microscopic findings on kidneys of group 3 (infected) rabbits were similar to those described for group 1, and the same diagnosis was made. One animal had noticeable fluid in the peritoneal cavity, and a second had approximately 30 ml of fluid present. The kidneys of all group 2 (broth) and group 4 (untreated) animals showed no obvious gross or microscopic abnormalities nor was any fluid present in the abdominal cavities of these animals.

ASLO titrations done on serum obtained at postmortem showed ASLO levels of less than 40 units per ml in all experimental and control animals. Three rabbits were infected with strain H8 and treated with penicillin as shown in table 1, group 3. Blood pressure readings and urinalyses were not done on this group of rabbits, since they were bled by cardiac puncture every 4 days. ASLO titrations were done on the serum with results shown in table 4. The ASLO titers on one animal showed a moderate increase 8 and 12 days after infection, reached a peak at 16 days and dropped rapidly by 20 days. The other 2 rabbits in the group showed a very rapid increase between 12 and 16 days after infection and both dropped rapidly by 20 days. The peak ASLO level occurred at almost the same time after infection as

TABLE 5.—*Treatment schedule of rabbits infected with* Str. viridans, M. pyogenes *and* Esch. coli.

Day	Group 5	Group 6	Group 7	Group 8
1– 4	Control period for all groups			
5	0.1 ml *M. pyogenes**	0.1 ml *Str. viridans**	0.1 ml *Esch. coli**	Untreated throughout experiment
15	80,000 units penicillin	80,000 units penicillin	80,000 units streptomycin	
17	80,000 units penicillin	80,000 units penicillin	80,000 units streptomycin	
19	80,000 units penicillin	80,000 units penicillin	80,000 units streptomycin	

* Whole culture subcutaneously.

did the onset of hypertension.

In summary, the blood pressures of 4 animals receiving intravenous filtrate from a culture of type 12 streptococci all rose within 8 days and remained high. Three of 4 animals locally infected with type 12 streptococci showed a similar rise 17 days after infection. Albumin and red blood cells appeared in the urines of both groups, and on postmortem at the 39th day, both groups showed microscopic evidence of lower nephron nephrosis. Untreated animals and those injected intravenously with sterile broth were completely normal during the course of the experiment and on postmortem.

These experiments have been duplicated with 12 animals each treated as in group 1 above (filtrate) and group 3 above (infection), and 4 animals in each control group (2 and 4 above). The same strain (H8) of type 12 streptococcus was used throughout. All animals receiving intravenous filtrate developed severe or moderate hypertension in an average of 8 days after the first injection of filtrate. Hypertension was maintained for 20 to 25 days, then declined, with all animals showing normal blood pressure 35 days after the first injection. Ten of the 12 showed albuminuria (1+ or 2+) and microscopic hematuria, which persisted for 10 to 20 days. The 12 locally infected animals all developed hypertension of varying degree in an average of 18 days after infection. This was maintained for 8 to 10 days, and all blood pressures were normal 35 days after infection. Ten of the 12 rabbits developed both albuminuria and microscopic hematuria, while 2 showed only albuminuria. Once again, untreated rabbits and those receiving sterile broth intravenously did not develop hypertension or urinary abnormalities.

More recently, rabbits have been locally infected with five other type 12 strains, according to the schedule shown for group 3 in table 1, Three rabbits each were infected with the following strains: H3, 8, 35, 7–54 and 7–56. The blood pressures of 14 of the 15 animals rose from a normal of less than 30 mm Hg to between 35 and 50 mm Hg within 15 to 20 days after infection. Hypertension of varying degree persisted for 3 to 4 weeks. Urine findings were not so consistent as in the first 2 experiments. Only 3 animals showed microscopic hematuria and 10 of the 15 developed albuminuria. Again the untreated control group showed no hypertension or urinary abnormalities.

Other group A streptococci

There is evidence both for[7] and against[15–17] the association of strains other than type 12 with outbreaks of acute glomerulonephritis in man. An experiment was set up to determine if localized infection with types 3, 10, 17, 18 and 25 would induce changes in rabbits similar to those following type 12 infections. Methods were identical to those described for groups 3 and 4 in table 1. Three rabbits were infected with each type and, with the untreated controls, were observed for 40 days.

At no time did the blood pressure of any animal in either infected or control groups rise above 31 mm Hg. This is a striking difference from the marked and

15. Kelsey, H. and Scholes, F. V. 1941, Streptococcal types in scarlet fever: the association of change of type with complications and cross-infection. Med. J. Australia, **1**: 728–733.
16. Henningson, E. J. and Ernst, J. 1938, Milk epidemic of scarlet fever and angina, originating from a cow with mastitis due to Streptococcus pyogenes (Lancefield group A). J. Hyg. **38**: 384–391.
17. Bloomfield, A. L. and Rantz, L. A. 1943, An outbreak of streptococcic sore throat in an army camp. Clinical and epidemiologic observations. J.A.M.A. **121**: 315–319.

sustained hypertension observed in animals infected with type 12 strains. Urines showed occasional traces of albumin and red cells, but these findings were neither consistent nor marked in any one animal or group of animals. It is apparent that urine findings and particularly blood pressure changes differ greatly in rabbits infected locally with type 12 and non-type 12 streptococci.

Other bacteria

Cary[18] found that what he believed to be acute nephritis could be produced in rabbits by intravenous injection of a variety of bacteria. Faber and Murray,[19] on the other hand, found that massive intravenous infections of dead cells of *Str. viridans*, *M. pyogenes* and *Esch. coli* failed to produce nephritis in rabbits. Since our methods differed from those of both, freshly isolated strains of the same three organisms were used to infect rabbits subcutaneously according to the schedule shown in table 5.

Blood pressure readings during the 60 days of the experiment showed minor fluctuations up to 33 mm Hg. At no time did readings on the treated animals (fig. 6) exceed those of normal animals (fig. 7). The urine of one rabbit infected with *Str. viridans* showed a 1+ albumin persisting from the 10th to the 30th day after infection. Two other infected animals showed occasional albuminuria and a few red blood cells. The other 6 infected and all of the control animals showed consistently normal urines. One animal from each of the 4 groups was sacrificed after 40 days and the remainder after 60 days. Gross and microscopic examination of all kidneys showed no obvious abnormalities.

DISCUSSION

Our present concept of the role of hemolytic streptococci in acute glomerulonephritis[20] postulates that some substance elaborated by the bacteria combines with renal protein and alters it in an unexplained way to form a new antigen. The new antigen enters the circulation and induces the formation of antibodies, which in turn react with the altered renal protein. This hypothesis offers an explanation for the latent period between infection and the onset of nephritis, and for the absence of bacteria in the renal lesions. Since early in this century investigators have attempted to produce experimental animal nephritis which would throw some light on the pathogenesis of acute nephritis. Arnott and Kellar[21] in 1936 believed that at that time there was no proven way of producing glomerulonephritis in any way comparable with the disease in man. Ehrich et al[22] in 1952 stated that although renal diseases generally described as glomerulonephritis have been produced in animals by injection of homologous anti-kidney serum, the impact of these experimental studies on our concept of human nephritis has been negligible. Ehrich's view remains essentially true.

Epidemiological evidence for the predominant association of type 12 strepto-

18. Cary, W. E. 1924, Bacteriology of urine in nephritis and experimental bacterial nephritis. J. Infect. Dis. **34**: 599–607.
19. Faber, H. K. and Murray, V. 1917, Studies in glomerulonephritis. III An attempt to produce glomerulonephritis by repeated injections of bacteria. J. Exper. Med. **26**: 707–720.
20. Schwentker, F. F. and Comploier, F. C. 1939, The production of kidney antibodies by injection of homologous kidney plus bacterial toxins. J. Exper. Med. **70**: 223–230.
21. Arnott, W. M. and Kellar, R. J. 1936, The effect of renal denervation on the blood pressure in experimental renal hypertension. J. Path. & Bact. **42**: 141–154.
22. Ehrich, W. E., Forman, C. W. and Seifer, J. 1952, Diffuse glomerular nephritis and lipoid nephrosis. A.M.A. Arch. Path. **54**: 463–503.

cocci with sporadic cases and with epidemics of acute glomerulonephritis in man has been presented particularly by Rammelkamp and Weaver,[7] Manser and Wilson[4] and Reed.[10] This association apparently is not shared to any appreciable extent by other serological types of group A streptococci. It seemed very probable that type 12 streptococci produced a nephritogenic substance lacking in most other types. This supposition has been tested by infecting rabbits with type 12 and other strains to determine if a clinical picture similar to that of acute glomerulonephritis in man could be induced. An infection localized on the dorsum was analagous to the localized pharyngeal infection with streptococci which precedes the majority of human cases.

Experimental evidence obtained during this investigation demonstrates that it is possible to produce in rabbits a clinical picture resembling that of acute glomerulonephritis in man following infection with type 12 streptococci. It has not been possible to produce these changes in rabbits infected with other serological types of streptococci or in those infected with *Str. viridans*, *Esch. coli* or *M. pyogenes*.

The hypertension which developed in 26 of 30 rabbits infected with 6 strains of type 12 streptococci appeared within 15 to 23 days, with an average of 18 days, after infection. This is similar to the average interval between infection and onset of acute glomerulonephritis in man, although the interval may be as short as 3 or as long as 33 days, according to Addis.[23] Sixteen rabbits injected intravenously with type 12 culture filtrate all developed hypertension in an average of 8 days after the first dose. The difference in time of onset by these two methods cannot be explained, unless it is presumed that the nephritogenic substance was present in higher concentration in the filtrate than the amount produced in the localized area of low-grade infection. The urinary findings in rabbits treated with type 12 streptococcal culture or filtrate were similar to those seen in acute glomerulonephritis in man. While the degree of albuminuria and hematuria was seldom marked, it was constant in most animals. Gross hematuria was observed in only one infected animal. It is significant that none of the animals infected with other serological types of group A streptococci or with other bacteria showed any change in blood pressure or urinary findings during similar observation periods.

Antistreptolysin O (ASLO) titers were done at regular intervals on several animals infected with type 12 streptococci. Titers reached maximum levels of 333 to 833 units per ml about the 16th day, then decreased very sharply and remained low. It is interesting to note that the appearance of maxiumm ASLO titers corresponds quite closely to the time of onset of hypertension. Rabbits receiving intravenous filtrate showed only very slight and fleeting increases in ASLO titer. Since three one-ml doses of filtrate containing less than 1 unit of streptolysin O per ml were sufficient to initiate hypertension, it seems very unlikely that streptolysin O is concerned with the clinical picture observed either in these animals or the infected ones. It would appear, from the action of filtrate in animals, that the nephritogenic substance is elaborated into the medium.

The lesions seen microscopically in the kidneys of both infected and filtrate-treated rabbits indicate tubular rather than glomerular damage, although the latter usually is associated with hema-

23. Addis, T. 1948, Glomerular Nephritis, New York. The MacMillan Company,

turia. The very mild glomerulitis observed in a few animals was much less severe than the degree of hematuria would suggest. There is a possible explanation for this in the fact that acute glomerulonephritis is rarely fatal, and knowledge of renal pathology in this disease is based on examination of rare cases at postmortem. It is possible that the tubular lesions observed in our experimental animals may occur in the majority of cases and that in a small number of individuals whose immunological response is more acute, severe glomerular damage and death may result. On the other hand, the rabbit may respond to nephritogenic substance primarily in the tubules.

It will be shown in a later paper[24] that the response of rabbits to reinfection with type 12 streptococci is similar to that following a single infection. Further work is being done in an effort to discover the nature and mode of action of the nephritogenic substance elaborated by type 12 streptococci.

24. Reed, R. W. and Matheson, B. H. 1954, Experimental nephritis due to type specific streptococci. II. The effect of repeated exposure to type 12 streptococci. J. Infect. Dis. **95**: 202–212.

SUMMARY

Hypertension, microscopic hematuria and albuminuria have been produced in the majority of rabbits infected locally with strains of type 12 streptococci recovered from widely separated outbreaks of acute glomerulonephritis in man.

Intravenous injection of filtrates from these cultures induces the same clinical picture more rapidly.

Local infections with non-type 12 group A streptococi and with other unrelated bacteria failed to produce nephritis.

The possible significance of these findings in relation to acute glomerulonephritis in man is discussed.

EXPERIMENTAL NEPHRITIS DUE TO TYPE SPECIFIC STREPTOCOCCI

II. THE EFFECT OF REPEATED EXPOSURE TO TYPE 12 STREPTOCOCCI

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The relationship of type 12 streptococci to acute glomerulonephritis in man has been proved by the recent epidemiological studies of Rammelkamp and Weaver,[1] Manser and Wilson,[2] and Reed.[3] Each of these investigations has established that the disease is much more likely to follow infections with type 12 than with other serological types of group A hemolytic streptococci. Reed and Matheson[4] have shown in a previous paper that it is possible to produce in rabbits a clinical syndrome which consistently resembles the picture of acute glomerulonephritis in man. Three weeks following localized infection with type 12 streptococci recovered from epidemics or sporadic cases of nephritis, hypertension, albuminuria and hematuria developed in the majority of rabbits so treated. Rabbits similarly infected with other serological types of streptococci or with other bacteria failed to show any of the above symptoms. Intravenous injection of cell-free filtrate from type 12 cultures also produced clinical nephritis but the onset was more rapid. The syndrome followed a single infection with type 12 streptococci. It was considered of some interest to expose recovered animals to a second infection with type 12 streptococci or to a second series of intravenous injections of type 12 culture filtrate to determine whether the same clinical picture would appear or whether the animals were immune to the action of type 12 nephritogenic substance.

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3. Reed, R. W. 1953, An epidemic of acute nephritis. Canad. M.A.J. **68**: 448–555.
4. Reed, R. W. and Matheson, B. H. 1954, Experimental nephritis due to type specific streptococci. I. The effect of a single exposure to type 12 streptococci. J. Infect. Dis. **95**: 191–201.

MATERIALS AND METHODS

Type 12 streptococci of strain H8 recovered from a convalescent case of acute glomerulonephritis during the Nova Scotia epidemic of 1951–52[3] were used throughout. The methods used were identical with those described by Reed and Matheson[4] in a previous paper.

EXPERIMENTAL RESULTS

I. Primary treatment: infection

Thirteen rabbits from 4 to 5 lb in weight were observed for 4 days, during which normal blood pressures and urinalyses were established. Nine of these rabbits were then injected subcutaneously with 0.1 ml of whole culture and treated 10 days later with penicillin. Four rabbits served as untreated controls (group 4 below). Hypertension developed in 8 of the 9 treated animals 18 days after infection and in the 9th, 26 days after infection. All animals developed varying degrees of albuminuria and hematuria. Blood pressures of 8 re-

turned to normal 41 days after infection, and urines were essentially normal by this time. Ten days later the recovered animals were randomly divided into 3 groups of 3 which received secondary treatment. Group 1 was reinfected, while group 2 received 29 ml of culture filtrate intravenously over a 21-day period. Group 3 received no further treatment. Group 4 was untreated throughout the experiment. The treatment schedule for groups 1 to 4 is shown in table 1. Blood pressure and urine changes of individual groups will be discussed separately.

a. Group 1 (infection+infection).—Following reinfection on the 56th day, there was a gradual rise in blood pressure (fig. 1), which continued in rabbit #40 until it was killed on the 90th day. Blood pressures of the two remaining animals dropped to within normal limits by the 95th day and remained there until the end of the experiment. All three animals, whose urines had returned almost to normal following first infection, developed albuminuria and hematuria of greater severity than in the first instance and, in addition, excreted many hyaline and granular casts until sacrificed on the 90th and 125th days, respectively. Table 2, column 1, shows the results of urinalyses of one of the three animals and is representative of changes observed in the entire group.

Kidneys of all animals were examined microscopically. The collecting tubules showed almost complete recovery from a degenerative process. Tubules in the boundary zone were conspicuously dilated. Distal nephrons showed marked dilatation, especially in the second convoluted tubule and in the loops of Henle. There was increased cellularity of some glomeruli, and slight digitation of a few capillary tufts was noted. The pathological diagnosis indicated almost complete recovery from lower nephron nephrosis with occasional very mild glomerulitis. At the time of postmortem, one kidney was removed from each animal aseptically and sliced into flasks of sterile broth, which were then incubated at 37 C for 4 days. Repeated subcultures failed to demonstrate growth from any of these kidneys.

b. Group 2 (infection+filtrate).—All these rabbits showed increasing hypertension which appeared 11 days after the start of filtrate injections (fig. 2) and persisted until the 90th, 110th and 125th days of the experiment. Following first treatment (infection) 3 animals developed albuminuria, and 2 hematuria, which persisted to the 40th day. Three weeks after the beginning of second treatment (filtrate), the urine of all animals showed albumin, red cells, cylin-

TABLE 1.—*Dosage schedule of infected rabbits re-exposed to type 12 streptococci or to filtrate.*

Day	Group 1	Group 2	Group 3	Group 4
1– 4	Control period for normal obeervations			
5	0.1 ml type 12 streptococci (H8) subcutaneously			
15	80,000 units penicillin intramuscularly			
17	80,000 units penicillin intramuscularly			
19	80,000 units penicillin intramuscularly			
55		3 ml filt. IV	No further treatment	Untreated throughout experiment
56	0.1 ml culture SC			
58		3 ml filt. IV		
61		3 ml filt. IV		
63	80,000 U pen. IM			
64		5 ml filt. IV		
65	80,000 U pen. IM			
67		5 ml filt. IV		
68	80,000 U pen. IM			
70				
72		5 ml filt. IV		
76		5 ml filt. IV		

SC = subcutaneous.
IM = intramuscular.
IV = intravenous.

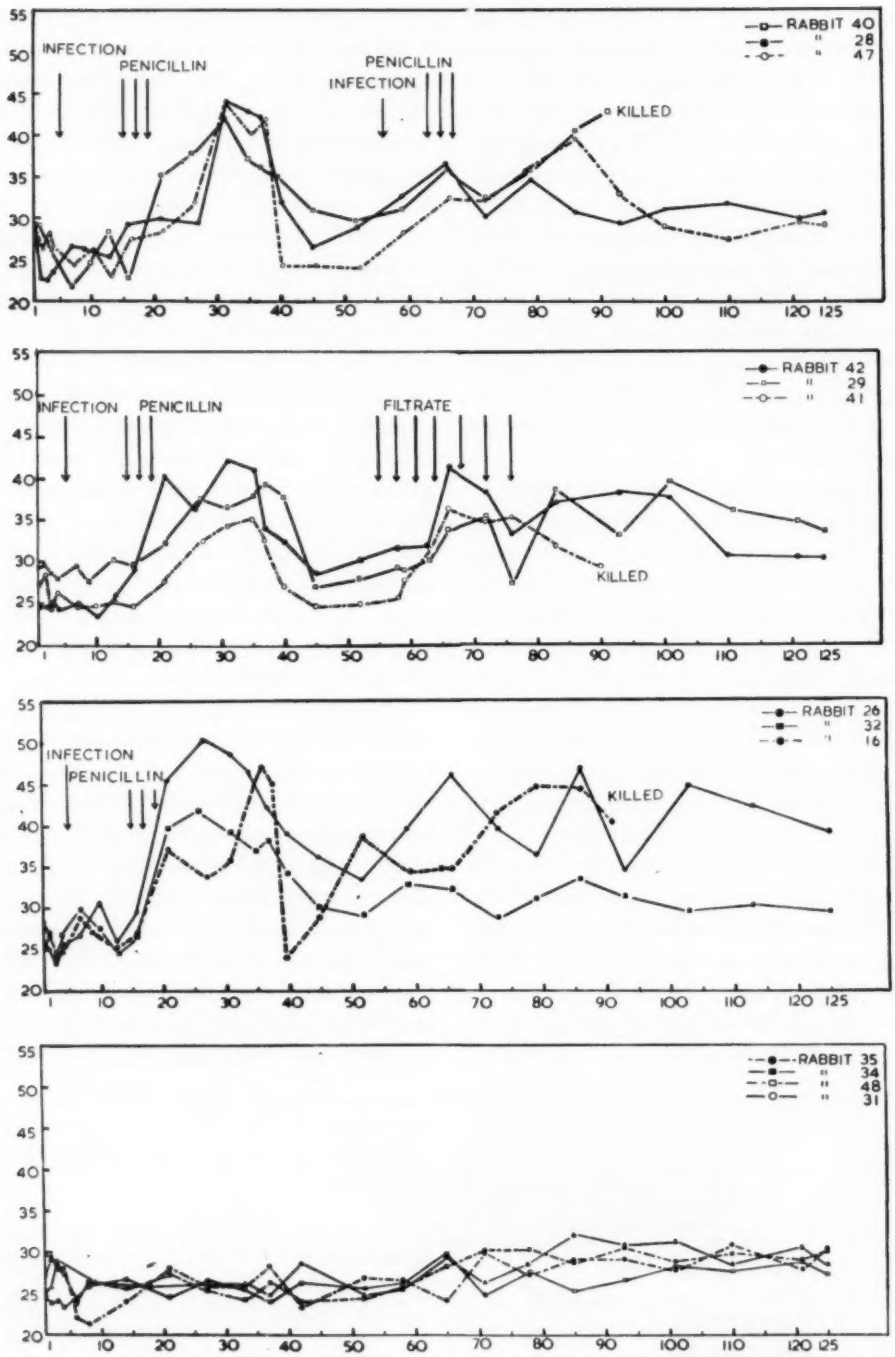

FIG. 1–4.—Peripheral arterial blood pressure in mm Hg (ordinate) plotted against time in days (abscissa) of groups of rabbits treated as follows:

FIG. 1.—(Top) Rabbits of group 1. Infection+infection.

FIG. 2.—(Top center) Rabbits of group 2. Infection+intravenous filtrate.

FIG. 3.—(Lower center) Rabbits of group 3. Infection+no further treatment.

FIG. 4.—(Bottom) Rabbits of group 4. No treatment.

droids and moderate numbers of granular and hyaline casts, which persisted until the end of the experiment. These findings were very similar to those of the group 1 animals. Table 2, column 2, shows urinalyses of one animal, and is representative of the entire group.

One rabbit was sacrificed after 90 days and 2 after 125 days. The kidneys of all animals were pale in color and rather soft. Microscopically the collecting tubules showed debased epithelium with evidence of regeneration. The convoluted tubules were dilated and the nuclear pattern in the epithelium was quite irregular. Numerous glomeruli were dilated and congested with red blood cells, and early digitation of capillary tufts was noted in a few glomeruli. Once again a diagnosis of lower nephron nephrosis was made.

c. Group 3 (infection+no treatment).—Blood pressures of 2 of the 3 rabbits returned to normal following primary infection. Two then developed hypertension a second time (fig. 3) although no further treatment was administered. Both showed considerable fluctuation of blood pressures between 35 and 45 mm Hg, which remained high until the animals were sacrificed on the 90th and 125th days. The blood pressure readings of rabbit #32 fluctuated from 30 to 35 mm Hg, a very moderate hypertension. All three animals excreted albumin and red cells at some time in the month following first infection. During the next three months all showed occasional albuminuria and at times 2 excreted small numbers of red cells. Towards the end of the experiment all excreted a few granular casts. The results of urinalyses of one of these three animals are shown in table 2, column 3, and are representative findings. Urine changes in this group are not so striking as in those receiving a second infection or intravenous filtrate.

TABLE 2.—*Representative urinalyses on rabbits in groups 1 to 3. Primary treatment: local infection with* Str. pyogenes (*H8*).

Days	Group 1 Rabbit #29				Group 2 Rabbit #47				Group 3 Rabbit #26		
	Alb.	RBC	G. C.	Misc.	Alb.	RBC	G. C.	Misc.	Alb.	RBC	Misc.
0–19*	0	—	—	—	0	—	—	—	0	—	—
21	+	—	—	—					+	46	—
27	+	—	—	—	+	—	—	—	+	10	—
31	0	64	—	—	+	55	—	—	0	22	—
37	0	—	—	—	4+	3100	—	—	0	—	—
41	—	16	—	—	+	31	—	—	+	31	—
44	+	—	—	—	0	—	—	—	+	—	—
51	0	—	—	—	0	—	—	—	+	—	—
55	+	—	—	—	0	—	—	—	0	—	—
57	2+	—	—	—	0	—	—	—			
63	+	160	125	—	+	—	—	—	0	—	—
70	+	—	27	—	2+	—	—	—	+	632	—
76	0	—	48	32 HC	+	48	192	24 PC	+	—	41 GC
83	+	—	—	43 HC	2+	276	4,968	552 HC 276 Cyl	+	28	9 GC
86	2+	—	150	150 HC 60 Cyl	3+	864	3,240	108 HC 432 Cyl			
90	+	306	30	—	+	—	21,000	20 HC	+	66	—
96	2+	—	66	—	2+	—	14,000	140 HC 280 Cyl			
100	+	920	138	—							
104	+	—	—	—	2+	—	6,400	320 Cyl	+	—	—
108	2+	—	34	—	2+	—	160	16 Cyl 80 PC			
113	+	—	—	—	+	—	105	21 Cyl	0	—	—
119	0	112	56	56 HC							
125	+	270	—	27 Cyl 108 HC	+	—	152	76 Cyl	0	—	—

Alb. = albumin.
RBC = red blood cells.
Cyl = cylindroids.
G. C. = granular casts.
HC = hyaline casts.
PC = pus cells.
All figures are expressed in 1000's.
* Urines of all rabbits examined twice in this period.

Microscopically, all kidneys showed irregular nuclear arrangement in the epithelium of convoluted tubules, with evidence of recovery from a degenerative process. Glomeruli were essentially normal. The pathological diagnosis was almost complete recovery from lower nephron nephrosis.

d. Group 4 (controls.)—Blood pressures remained under 30 mm Hg for the first 2 months but tended to increase slightly as the rabbits increased in age and weight (fig. 4). The urine of each of the 4 animals was examined 18 times during the course of the experiment and at no time were abnormal constituents in evidence. One rabbit was sacrificed on the 90th day and 2 on the 125th day. The kidneys of all animals were normal both in the gross and microscopically.

e. Summary of results.—All 9 rabbits developed hypertension an average of 18 days following primary infection, and 8 of the 9 had normal blood pressures 3 weeks later. All rabbits developed albuminuria and 8 of them varying degrees of hematuria. Those receiving either infection or intravenous filtrate as a second treatment developed secondary hypertension accompanied by very marked urinary changes. Animals receiving no further treatment also showed secondary hypertension with only mild urine changes. Untreated animals showed neither hypertension nor urinary abnormalities. The kidneys of all treated animals showed evidence of recovery from lower nephron nephrosis, regardless of what second treatment was administered. Kidneys of untreated animals were normal microscopically.

II. Primary treatment: intravenous filtrate

Sixteen rabbits were observed for a 4-day period for the recording of normal blood pressures and urinalyses. Twelve animals then received increasing doses of cell-free filtrate intravenously, while 4 received sterile Todd-Hewitt broth by by the same route and in the same dosage. Hypertension appeared in all of the filtrate-treated animals within 10 days after the first injection. Red cells and albumin were excreted by most of these animals from the 20th to the 40th day. Both blood pressure and urine findings were normal by the 45th day in most animals. Two rabbits died during this period. The group receiving sterile broth broth showed no changes.

On the 56th day, the surviving treated animals were randomly divided into 2 groups of 3 and one group of 4 for second treatment. Group 5 had been given continuous injections of filtrate (31 ml), group 6 were infected subcutaneously with whole culture, and group 7 received no further treatment. Group

TABLE 3.—*Dosage schedule for filtrate-treated rabbits re-exposed to filtrate or to type 12 infection.*

Day	Group 5	Group 6	Group 7	Group 8
1– 4		Period for normal observations		
5		1 ml filtrate intravenously		1 ml broth IV. Further doses as for group 5
8		1 ml filtrate intravenously		
11		1 ml filtrate intravenously		
14		2 ml filtrate intravenously		
17		2 ml filtrate intravenously		
20		3 ml filtrate intravenously		
27–69	3 ml filt. IV at weekly intervals		No further treatment	3 ml broth IV at weekly intervals
56		0.1 ml cult. SC		
63		80,000 U pen. IM		
65		80,000 U pen. IM		
67		80,000 U pen. IM		

SC = subcutaneous.
IV = intravenous.
IM = intramuscular.

8 comprised the broth control rabbits. The treatment schedule for these animals is shown in table 3. One animal in each group was sacrificed after 90 days, the remainder after 125 days. Findings in each of these groups will be considered separately.

a. Group 5 (continuous filtrate).—Hypertension developed in all 3 rabbits within 10 days after the first injection of filtrate and was maintained at moderately high levels continuously until the 65th day (fig. 5). At this point, 1 week before the final injection of 3 ml of filtrate, blood pressures decreased and reached normal levels in 2 rabbits by the 85th day and in the 3rd by the 110th day. During the treatment period, albuminuria was observed almost continuously in all rabbits, but microscopic hematuria was not a notable feature at any time. In the post-treatment period all showed albuminuria, especially rabbit #38. Two excreted granular casts and cylindroids in moderate numbers but again hematuria was not severe. Table 4, column 1, shows the urine findings of rabbit #56 during the experiment.

The kidneys of two animals were somewhat pale while those of rabbit #56 were purplish and soft. The most conspicuous change seen microscopically was in the collecting tubules which showed debased epithelium and casts containing epithelial material. The loops of Henle and second convoluted tubules were dilated and showed evidence of both degeneration and regeneration. Glomeruli were congested with coagulum in the capsular space. A diagnosis of typical lower nephron nephrosis was made.

b. Group 6 (filtrate+infection).—All rabbits developed hypertension within 10 days after the first injection of filtrate. Blood pressures dropped to normal very precipitously betwen the 35th and 38th day (fig. 6). The blood pressures of 2 rabbits were rising when infection was initiated on the 56th day. Rabbit #21 remained fairly constantly at 35 mm Hg, while #33 fluctuated between normal and 35 mm. Rabbit #51 did not develop a secondary rise in blood pressure at any time. All animals showed albuminuria and low grade microscopic hematuria frequently up to 55 days. Following infection, all three rabbits showed continuous albuminuria and hematuria. In addition, all excreted many granular and hyaline casts and cylindroids from the 77th day to the end of the experiment. Table 4, column 2, shows urine findings of rabbit #21 which are representative of the group. The urine changes in #51, which did not show the second blood pressure increase, were just as severe as those shown in the table for #21.

The kidneys showed more severe microscopic changes than those of group 5. Collecting tubules were dilated, with debased epithelium and casts in the lumen. The second convoluted tubules and loops of Henle were greatly dilated. The diagnosis was healing lower nephron nephrosis.

c. Group 7 (filtrate+no further treatment.)—Six animals developed marked increases in blood pressure 10 days after filtrate injections began (fig. 7). Two in particular, #45 and #50, showed a rise from 28 mm to 42 mm in this period. Rabbit #50 died on the 22nd day. Pressures of 4 survivors reached normal by 38 days, and that of the 5th remained elevated. Rabbit #71 died on the 43rd day. From the 60th day, rabbit #45 showed a second rise in blood pressure, which reached a peak of 53 mm on the 82nd day and remained high until the 120th day. One other showed a mild increase of short duration, while two did not develop a second rise. All surviving animals had some degree of albuminuria and microscopic hematuria from the

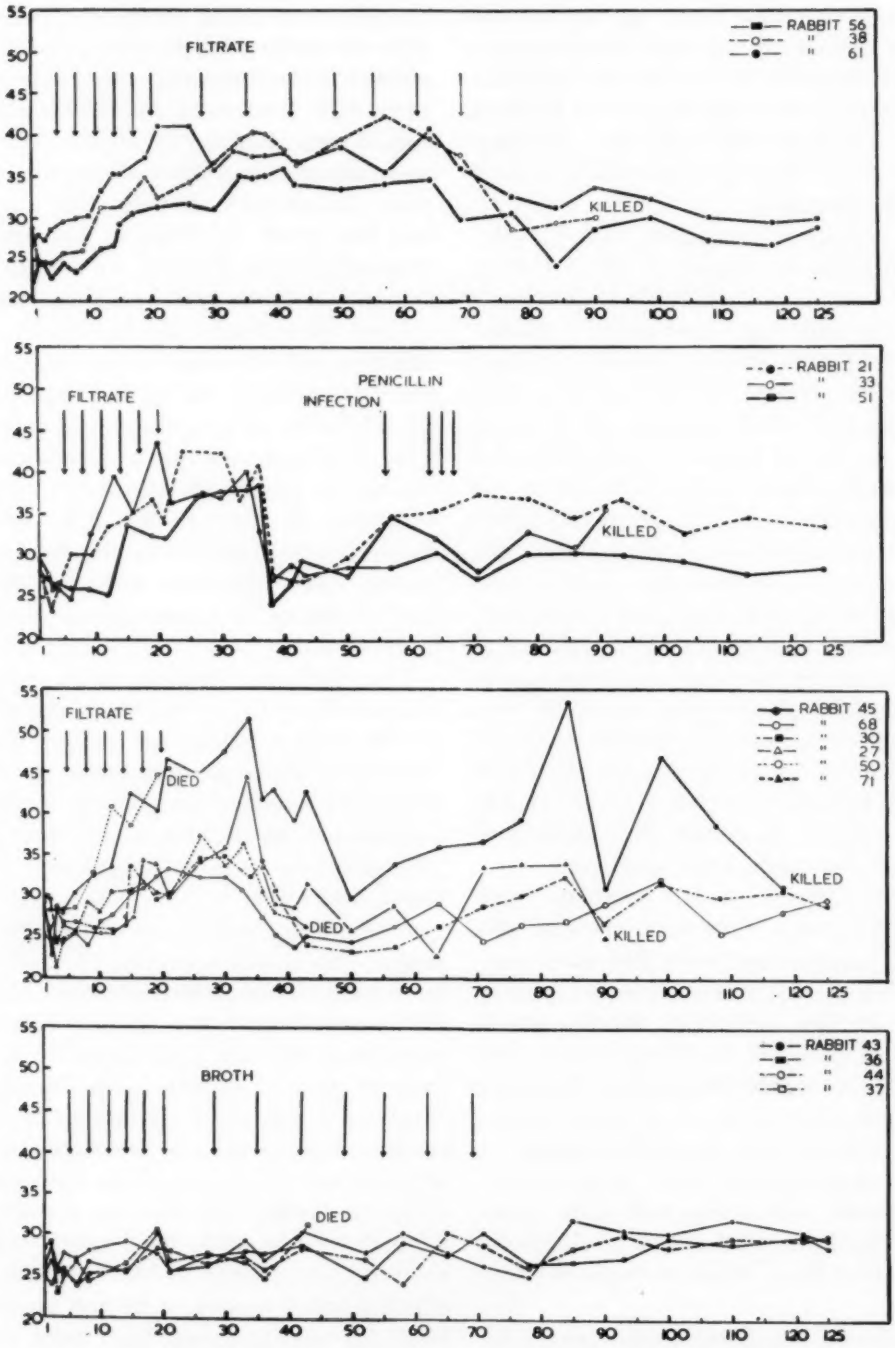

FIG. 5–8.—Peripheral arterial blood pressure in mm Hg (ordinate) plotted against time in days (abscissa) of groups of rabbits treated as follows:

FIG. 5.—(Top) Rabbits of group 5. Continuous intravenous filtrate.

FIG. 6.—(Top center) Rabbits of group 6. Intravenous filtrate+infection.

FIG. 7.—(Lower center) Rabbits of group 7. One course of intravenous filtrate+no further treatment.

FIG. 8.—(Bottom) Rabbits of group 8. Continuous sterile broth intravenously.

TABLE 4.—*Representative urinalysis on rabbits in groups 5 to 7. Primary treatment: intravenous injection of streptococcal filtrate.*

Days	Group 5 Rabbit #56				Group 6 Rabbit #21				Group 7 Rabbit #45		
	Alb.	RBC	G. C.	Misc.	Alb.	RBC	G. C.	Misc.	Alb.	RBC	Misc.
0–29*	0	—	—	—	0	—	—	—	0	—	—
30	+	60	—	—	+	10	—	—	2+	—	—
35	+	40	—	—	+	—	—	—	2+	96	—
38	+	—	—	—	+	—	—	—	2+	19	—
43	+	—	—	—	2+	—	—	—	2+	123	—
46	0	—	—	—	0	—	—	—	+	—	—
51	0	—	—	—	0	—	—	—	0	—	—
56	0	—	—	—	2+	—	—	—	0	—	—
64	+	31	12	—	+	—	—	—	0	—	—
72	0	—	—	—	+	56	28	—	0	16	—
78	+	—	—	—	+	50	20	25 PC			
83	0	—	—	—	2+	—	240	—	+	150	—
86	0	—	—	—	2+	696	464	464 PC			
91	+	—	—	36 Cyl	+	—	276	460 HC 184 Cyl	+	—	—
98	0	96	48	24 Cyl	2+	141	282	225 HC 225 Cyl	+	—	128 Cyl
104	+	—	80	32 Cyl	2+	208	312	164 HC 52 Cyl			
109	+	—	—	26 Cyl	2+	36	54	1800 PC 72 Cyl	2+	150	60 GC 90 Cyl
114					+	169	84	42 HC 42 Cyl			
120	+	—	—	—	+	640	64	64 HC 128 Cyl			
125	+	—	—	—					0	—	—

Alb. =albumin.
RBC =red blood cells.
Cyl =cylindroids.
G. C. =granular casts.
HC =hyaline casts.
PC =pus cells.
All figures expressed in 1000's.
* Urines of all rabbits examined 4 times in this period.

20th to the 90th day. In addition, two excreted a few hyaline and granular casts and cylindroids from the 90th to the 125th day. Table 4, column 3, presents the urine findings of rabbit #45, which showed the greatest degree of hypertension. Others, especially #30, had more marked urinary abnormalities.

The kidneys of #27, sacrificed on the 90th day, showed dilated collecting tubules with casts and debased epithelium. The convoluted tubules were also dilated and showed evidence of regeneration. Glomeruli were congested with coagulum in the capsular space and occasional capillary tufts demonstrated slight digitation. Recovering lower nephron nephrosis was the diagnosis. The kidneys of rabbit #45, which had mild urine changes but marked hypertension, showed no marked abnormalities.

d. *Group 8 (broth controls).*—None of the 4 animals in this group showed any increase in blood pressure (fig. 8) compared to the untreated control group (fig. 4). Except for #44, which had a 1+ albumin on the 19th day and 10,400 red cells on the 39th day, the urines of all rabbits remained normal during the entire experiment. Microscopic examination of all kidneys showed no obvious abnormalities.

e. Summary of results.—All rabbits on a single course of intravenous injections of filtrate developed hypertension 10 days after the first injection, which subsided in most by the 40th day. Those animals receiving continuous injections for 3 months maintained hypertension during that period. Rabbits receiving no further treatment or infected on the 56th day developed mild to severe hypertension at almost the same time. Most treated animals had some degree of albuminuria and microscopic hematuria persisting from 20 to 50 days. This did not increase markedly in those re-

TABLE 5.—*Antistreptolysin O titers of animals re-exposed to type 12 infection or filtrate.*

Group	Treatment	Rabbit no.	Day					
			46	63	70	90	106	125
1	Infection	28	12–50	<12	<12	—	<12	<12
	+infection	40	<12	<12	<12	<12*	—	—
		47	<12	<12	<12	—	<12	<12*
2	Infection	41	12–50	12–50	12–50	12–50*	—	—
	+filtrate	29	<12	125–166	50–100	—	12–50	12–50*
		42	<12	<12	<12	—	<12	<12
6	Continuous	38	<12	—	12–50	12–50*	—	—
	filtrate	61	<12	—	12–50	—	<12	<12
		56	<12	—	12–50	—	12–50	12–50*
7	Filtrate	33	<12	125–166	50–100	12–50*	—	—
	+infection	21	<12	100–125	125–166	—	12–50	<12*
		51	<12	500–625	1250–2500	—	12–50	<12

* Date killed.

ceiving continuous injections or no further treatment. However, urine changes were severe in those infected after receiving filtrate. The kidneys of most treated animals showed in varying degree the changes associated with lower nephron nephrosis. No abnormalities were observed in rabbits injected intravenously with sterile Todd-Hewitt broth.

III. Antistreptolysin O titers

It has been shown[4] that a single infection with type 12 streptococci resulted in a marked increase in ASLO titer 16 days following infection but which dropped rapidly. ASLO titrations were done on a number of animals exposed to type 12 streptococci or their products a second time. The results are shown in table 5. Animals of group 1 which were reinfected showed no increase in ASLO titer from the 46th day to the end of the experiment. Of three rabbits which received filtrate following infection (group 2), only one showed an appreciable increase in ASLO. Group 6 animals receiving continuous injections of filtrate showed no change in titer. Two of those infected following filtrate injections showed moderate increases in ASLO titer, while the third reached a peak of 1250 to 2500 units per ml 2 weeks after infection. All the above rabbits developed two or more clinical signs of nephritis regardless of whether ASLO titers increased or not.

DISCUSSION

It has been shown by Reed and Matheson[4] that rabbits will develop clinical acute glomerulonephritis following localized infection with type 12 streptococci or intravenous injection of culture filtrate. Rabbits similarly treated have developed the same symptoms and appear to recover. Almost without exception, however, these rabbits showed a recurrence of hypertension, albuminuria and hematuria about 8 weeks after first exposure, whether or not they were re-exposed to type 12 streptococci or their products. All animals developed some degree of hypertension, but urine changes were more severe and more prolonged in rabbits treated a second time than in those not so treated. This was so especially when secondary treatment was localized infection.

Microscopic examination of kidneys of all type 12 treated animals showed mainly tubular damage of the type associated with lower nephron nephrosis. Glomerular changes in most cases consisted of dilation and congestion of capillaries. Slight digitation of capillary tufts was observed in a few cases. These changes were much less severe than would be expected from the severity of the urine changes and hypertension.

While the pathological findings in most kidneys were comparatively mild, there can be no doubt concerning the clinical findings. The time of onset of hypertension, albuminuria and hematuria in rabbits following infection is completely analogous to the average time of onset of the same symptoms in man following streptococcal pharyngitis. It is apparent that the first exposure to type 12 streptococci or their products did not result in immunity to the action of the nephritogenic substance.

It is of some interest to speculate on the possible nature of the nephritogenic substance (NS) which is elaborated by type 12 streptococci and apparently not by most other types. Since cell-free filtrate induces the same symptoms as infection, NS is apparently a substance which leaves the cell freely. It is rather attractive to assume that NS is a protein and an exotoxin. If this is so, then there is some evidence to suggest its possible nature. It is known that types 10 and 12 share a common M-substance, while their respective T-substances differ.[5] We have demonstrated that infections with several type 12 strains will induce clinical acute nephritis while infection with type 10 will not.[4] This circumstantial and limited evidence would support the hypothesis that type 12 NS and type 12 T-substance are identical. If other serological types could be found which share a common T-substance with type 12 and which induce clinical acute nephritis in infected rabbits, this would be strong support indeed. It is rather difficult, however, to accept NS as an exotoxin with a particular affinity for renal protein.

The present hypothesis for the pathogenesis of acute glomerulonephritis presumes that NS combines with renal protein to form a complete antigen which is then thrown into the circulation and induces formation of antibodies against renal protein. This theory almost requires that NS be a nonprotein and a haptene. One can fit a haptene NS into such a hypothesis much better than a toxic antigenic protein. A haptene NS readily explains the first attack of acute nephritis about 3 weeks after first exposure when antibody production is at its peak. It may also partially explain the observations on animals re-exposed to NS either as infection or as filtrate. Severe urine changes are undoubtedly due to further renal damage superimposed on a kidney still recovering from the damage suffered during the first attack of acute nephritis. It is more difficult to explain the recurrence 8 weeks after urinary exposure with no further treatment.

The haptene nature of NS has been tentatively indicated by recent experiments in this laboratory. Further studies of NS are currently in progress to determine its chemical nature and possible mode of action.

SUMMARY

It has been demonstrated that rabbits exposed to type 12 streptococcal infections or filtrates develop clinical signs of acute glomerulonephritis about 3 weeks later, appear to recover, then show the same clinical signs again 8 weeks after primary exposure whether or not they are re-exposed. Urine changes are much more severe in animals re-exposed to type 12 streptococci or their products.

Microscopically the kidneys of all treated and retreated animals showed

5. Watson, R. F. and Lancefield, R. C. 1944, Studies on the antigenic composition of group A hemolytic streptococci. III. Types with serologically identical M but distinct T antigens: types 10 and 12. J. Exper. Med. **79**: 89–98.

mainly tubular damage of the type associated with lower nephron nephrosis. Glomerular damage was minimal, consisting largely of dilation and congestion of capillaries with slight digitation in a few cases.

The nature of type 12 nephritogenic substance was discussed in the light of these findings and the present hypothesis concerning the pathogenesis of acute glomerulonephritis.